
THE SECRET
RECIPE OF
HUS
JOYCE GEELEN

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The pathogenesis of haemolytic-uraemic syndrome: a secret recipe

Een wetenschappelijke proeve
op het gebied van de
Medische Wetenschappen

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The mind is always telling you
that you need something else to be happy.
This is not the truth
you need nothing to be happy.
Happiness is your nature.
When you are just in this moment
you see that nothing is missing.
You have it all!

Buddhatobuddha

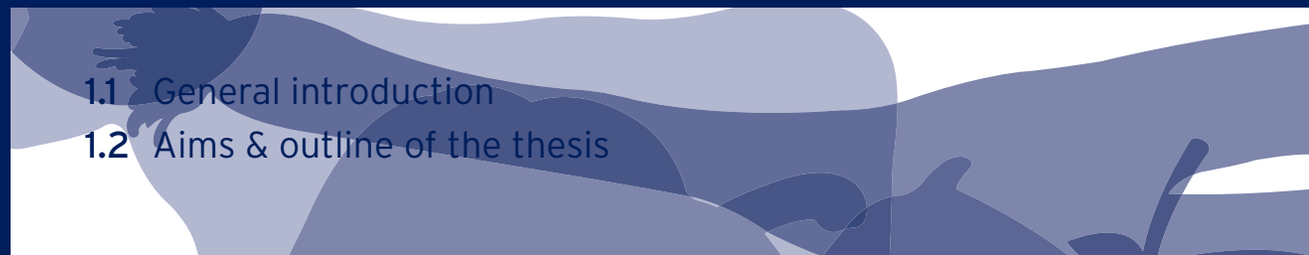
For Professor

Contents

Abbreviations

A/E	Attaching and Effacing
AP-1	Activator Protein-1
BF	Factor B
CCP	Complement Control Protein
CFHR	Complement Factor H Related proteins
CSF-1	Colony Stimulating Factor-1
D+HUS	Diarrhoea-associated Haemolytic Uraemic Syndrome
DRM	Detergent Resistant Membranes
EDTA	Ethylenediaminetetra-acetic Acid
EHEC	Enterohaemorrhagic *Escherichia coli*
ECP	*Escherichia coli* Common Pilus
ER	Endoplasmic Reticulum
ESC	*Escherichia coli* Secretion Apparatus
ESP	*Escherichia coli* Secreted Proteins
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanide
Gb3	Globotriaosylceramide
GMVECs	Glomerular Microvascular Venous Endothelial Cells
HBSS	Hanks Balanced Salt Solution
HD	Haemodialysis
HEP	Heparitinase I
HF1	Factor H
HPTLC	High Pressure Thin-Layer Chromotography
HS	Human Serum
HSA	Human Serum Albumin
HS	Heparan Sulfate
HSPG	Heparan Sulfate Proteoglycans
HUS	Haemolytic Uraemic Syndrome
HUVECs	Human Umbilical Venous Endothelial Cells
IEC	Intestinal Epithelial Cell
IF	Factor I
IL	Interleukin
LEE	Locus of Enterocyte Effacement
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MAPK	Mitogen Activated Protein Kinase-system
MCP	Membrane Cofactor Protein
NBCS	New Born Calf Serum
NF-κB	Nuclear Factor Kappa B
PBS	Phosphate Buffered Saline
PD	Peritoneal Dialysis
PE	R-phycoerythrin
PMN	Polymorphonuclear leukocytes
RCA	Regulators of Complement Activation-locus
RNA	Ribonucleic Acid
SAP	Serum Amyloid P
SCR	Short Consensus Repeats
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate- Poly Acrylamide Gel Electrophoresis
SNP	Single Nucleotide Polymorphism
STEC	Shiga-like Toxin producing *Escherichia coli*
STP	Serine, Threonine and Proline-rich domain
Stx	Shiga-like toxin
StxB	Shiga-like toxin B-subunit
TCA	Trichloroacetic Acid
TGN	Trans-Golgi Network
TIR	Translocated Intimin Receptor
TNF-α	Tumor Necrosis Factor Alpha
TTSS	Type Three Secretion System
VWF	Von Willebrand Factor
WPbs	Weibel Palade bodies

Chapter 1
General introduction, aims & outline of the thesis



‘Until now, the mechanism of disease is partially ‘a secret recipe’.

Chapter 1.1
Introduction

1 The disease

The haemolytic-uraemic syndrome (HUS) is a clinical trias consisting of micro-angiopathic haemolytic anaemia, thrombocytopenia and acute renal failure (1). There are various underlying pathogenic mechanisms (2). Two of the most occurring causes are infection-induced and disorders of complement regulation (non-Stx HUS). Both mechanisms will be explained in this introduction. It can also be found with defective cobalamine metabolism, quinine-induced or associated to clinical entities like HIV, malignancies, transplantation and auto-immune diseases.

There are no proven effective therapies to arrest the devastating renal damage induced by both inflammation and coagulation. Since specific treatment is still lacking, more knowledge is urgently needed. Until now, the mechanism of disease is partially ‘a secret recipe’.

2 Infection-induced HUS

This cause of HUS is often preceded by (bloody) diarrhoea. This clinical feature combined with the classical elements of HUS trigger paediatricians to consider the diagnosis of infection-induced HUS.

2.1 The bacteria

Different pathogens can cause HUS. The most abundantly isolated organism in approximately 50% of patients with HUS is *Escherichia coli* with serotype O157:H7 (O: type of LPS and H; type op flagellum) (3, 4). Manning *et al* discovered by SNP-analysis nine different clades (group of micro-organism originating from an identical ancestor) of which clade 8 has acquired critical factors that contribute to more severe disease (5). However, other serotypes of *Escherichia coli* are increasingly identified like O26:H11, O91:H21, O103:H2, O111:H8, O113:H21, O145:H28/H25 and O157:non-motile (6, 7). This underlines the rising importance of non-O157:H7 serotypes, although patients with O157:H7 serotypes require dialysis for a longer time and had bloody diarrhoea more often (8). Also *Streptococcus pneumoniae* can be the causative micro-organism in HUS (9). This pathogen releases neuraminidase which leads to exposure of the Thomsen-Friedenreich crypt-antigen, resulting in the clinical picture of HUS. This thesis is focusing on *Escherichia coli* O157:H7 which is a Gram-negative anaerobic micro-organism. The most important virulence factor of this bacterium is the synthesis of Shiga-like toxin (Stx). This type of *Escherichia coli* is therefore called: Shiga-like toxin-producing *Escherichia coli* (STEC). Other important virulence factors are the presence of the gene for a specific type of Stx, called Stx2 and the intimin-protein encoding eae-gene (10).

This bacterium can be detected as a normal commensal in the colon of cattle and domestic animals like sheep, pigs, goats and dogs. It will reach the human food chain from these animal sources, mainly by contamination of meat in slaughter houses (especially ground beef) or other food products that could have been in contact with animal droppings (dairy products, fruit and vegetables). But also water can be contaminated and person-to-person transmission has been described (11). The last break-out in The Netherlands was most probably caused by contaminated ‘steak tartar’ (12). Recently, Heuvelink *et al* described the incidence of STEC on petting farms (13). The typical course of infection-induced HUS is onset of diarrhoea which becomes bloody in 90% of patients. In 85% there will be spontaneous resolution, but 15% will develop HUS (1). This occurs especially in younger children and elderly. It is still not clear why these age groups have a predisposition.

2.2 The toxin

Stx is an exotoxin consisting of an AB5 structure. The A- and B-subunits are non-covalently bound. The B-subunits can bind the toxin, whereas the A-subunit is the enzymatically active part of the toxin (14). The toxin can be internalised into target cells, after binding to a specific receptor, called globotriaosylceramide-receptor (Gb3, or CD77) (15). This receptor can be detected in the kidney on glomerular endothelial cells and cortical tubules and does not vary with age (16).

The A-subunit is cytotoxic because it disables ribosomes. It functions as RNA N-glycosidase. In the cytosol, it removes one adenine from the 28S rRNA of the 60S ribosomal unit and thereby inhibits the elongation factor 1-dependent aminoacyl-tRNA binding to the ribosome (17, 18). This will lead to an

inhibition of peptide elongation and consequently an overall inhibition of protein synthesis in target cells like the renal glomerular endothelial cells (19). There are two different families of Stx, called Stx1 (with variants Stx1c and Stx1d) and Stx2 (with variants Stx2c, Stx2d, Stx2d- and Stx2e). Especially STEC producing Stx1, Stx2 and Stx2c can be isolated from HUS-patients (7). However, also *Escherichia coli* O157 strains which did not produce Stx were detected in patients with HUS (20). This can be inherently Stx-negative strains or strains which loose the Stx gene during infection (21). This initiated an intensive search for other non-Stx virulence factors that can play a role in the pathogenesis of HUS. Discovered candidates are cytolethal distending toxin (22), EHEC haemolysin (23), and subtilase cytotoxin (24). These novel proteins are discussed in chapter 5. More research is necessary to understand their role in HUS.

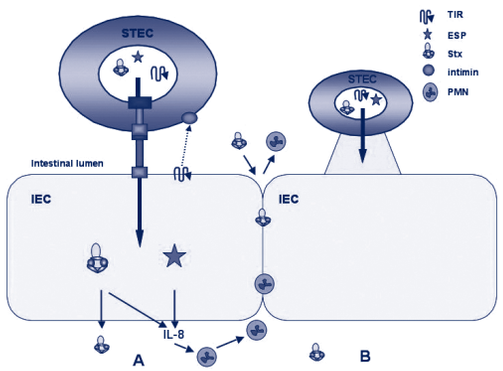
2.3 The journey of Stx

STEC is a non-invasive bacterium which has fascinating mechanisms to transport Stx across the intestinal barrier and into the circulation in order to reach its main target, the renal endothelium.

2.3.1 Passage of Stx across the intestinal wall

After surviving the acidity of the stomach, the ingested STEC will adhere to intestinal epithelial cells (11). It was generally assumed that the colon and perhaps also the distal ileum were the preferred sites for colonization. The human intestinal epithelial cells (IEC) do not have a specific receptor for STEC. Rendon *et al* demonstrated with adherence assays combined with mutagenesis that STEC can use a pilus (polymeric adhesive fiber) called *E coli* common pilus (ECP) for the facilitation of initial attachment and subsequent colonization (25). After adherence, STEC H7 flagellin can interact with Toll-like receptor 5 on IEC which directly up-regulates its production of pro-inflammatory chemokines (26). Moreover, the micro-organism is able to insert a 'home-made' receptor into the IEC. To achieve this, STEC must carry the genetic information to create attaching and effacing (A/E) lesions. Proteins involved in this lesion are encoded by genes on a pathogenicity island called the locus of enterocyte effacement (LEE) (27). STEC uses a type III secretion system (TTSS) to transfer its components to the IEC. This is a multi-component organelle assembled by a group of proteins, called ESC (*Escherichia coli* secretion apparatus) (28). After the TTSS is finished, microbial proteins (*Escherichia coli*-secreted proteins (ESP)) are released into it to produce a pore in the IEC. The pore will create an easy accessible pathway between STEC and IEC. To optimize the attachment, ESP are released into the IEC to initiate the A/E lesion. This will lead to aggregation of actin and rearrangements of the cytoskeleton, forming a pedestal and effacing the microvilli. To finally attach STEC, the translocated intimin receptor (TIR) is transported over the TTSS and inserted in the IEC. The receptor can bind to intimin which is present on the outer membrane of STEC (29). With this binding, STEC can closely adhere to the IEC.
The attachment of STEC to IEC is demonstrated in figure 1.

Figure 1 Attachment of Stx to IEC and passage of Stx



STEC adheres to the IEC. After assembly of the TTSS, the bacterium is able to insert microbial products like ESP and Stx into the cell. ESP induces the formation of a pedestal to optimize the adherence. This pedestal can be seen in B. Both Stx and ESP trigger the IEC to produce IL-8 which attracts polymorphonuclear leukocytes (PMN). Stx can cross the IEC both trans- and paracellular.

With the completed TTSS, Stx can be released into the IEC. Acheson *et al* demonstrated that biologically active Stx1 is capable of moving across intact IEC without cellular disruption (30). This occurred in an energy-dependent and saturable manner. Other data suggested that Stx is not just moving passively across the IEC, but also stimulates IEC to produce the chemokines like interleukin-8 (31). The group of Acheson also described that STEC interaction with IEC induces neutrophil recruitment to the intestinal lumen (32). This mechanism increases the paracellular permeability which in parallel increases the amount of Stx that passes into the underlying tissues. This will lead to an increased risk of HUS. It needs to be mentioned that the work on the transport of Stx is performed on colonic carcinoma cell lines, which carry Gb3-receptors. This receptor, however, was never detected on normal human colon. But, recent work from Schuller *et al* described experiments showing binding of Stx to Paneth-cells in both normal and inflamed intestinal tissue (33). These cells contain antimicrobial agents like α-defensins and lysozyme, and are specifically present in the small intestine. The presence of Gb3-receptors on Paneth cells could be detected with an antibody against Gb3. This new findings will need more research to investigate the functional aspect and possible involvement in the pathogenesis of STEC induced HUS.

2.3.2 Passage of Stx in circulation

After Stx reaches the circulation, it has to be transported to the renal endothelium. Stx was never detected in the blood of HUS-patients. Therefore, a carrier was suggested. Many possibilities have been discussed in the literature.

2.3.2.A Erythrocytes

Erythrocytes are the cells most abundantly present in the circulation. These red blood cells also carry a Gb3-receptor. Gb3 is the Pk-antigen of the P blood group system (15). This antigen is present on almost all types of erythrocytes, but can vary in expression. This indicates that erythrocytes could have a protective role in HUS by catching Stx out of the circulation. Taylor *et al* demonstrated that erythrocytes from patients with HUS express a weak or absent Pk-antigen (34). This was underlined by the work of Newburg *et al* that reported a lower amount of Gb3 on erythrocytes of children with HUS as determined with high-pressure liquid chromatography (35). The group of Bitzan *et al* confirmed the binding of Stx to human erythrocytes *in vitro* (36). However, Robson *et al* could not find a protective role of the Pk-antigen for the development of HUS (37). Also, te Loo *et al* could not measure a binding of Stx to erythrocytes (38). Until now, there are no indications that Stx uses erythrocytes as a carrier to reach the renal endothelium.

2.3.2.B Platelets

In patients with HUS, thrombocytopenia caused by platelet consumption is present. This indicates a role for platelets in the pathogenesis of HUS. If Stx can directly bind and activate platelets is extensively discussed. There are *in vitro* experiments that demonstrate the binding of Stx to platelets (39, 40, 41). Platelets were found to carry Gb3-receptors and Stx can also bind to a glycosphingolipid identified as band 0.03 specifically found on platelets (39). In the systemic circulation of HUS-patients, platelets are found to be activated and degranulated, and serum from HUS-patients induced adhesion and aggregation of normal platelets (42). Guessous *et al* demonstrated the ability of Stx to induce human microvascular endothelial cells to release chemokines and other factors that stimulate platelet function (43). However, other publications did not demonstrate a direct binding of Stx to platelets (38, 44).

2.3.2.C Polymorphonuclear leukocytes

Another possible carrier for Stx in the circulation is the polymorphonuclear leukocyte (PMN). Different groups demonstrated an increased activation of PMN in HUS with a damaging effect on the endothelium. This was demonstrated by changed markers of degranulation (an increase in α-1 antitrypsin complexed elastase in serum, decreased endothelial cell fibronectin, CD16- and CD11b-expression and intracellular granule content in PMN) and functional studies (a reduced degranulatory capacity and reactive oxygen species generation, an impaired antibody-dependent cellular cytotoxicity, and a more intense binding of PMN to endothelium) in patients with HUS (45, 46, 47,

48). This, again, raised the question whether Stx did have a direct or indirect effect on PMN. Finally, te Loo *et al* described in their work a selective binding and transfer of Stx to PMN both *in vitro* and *in vivo* (38, 49). Also, Brigotti *et al* could detect Stx bound to PMN in the blood of HUS-patients with flow cytometry (50). But in contrast to the work of te Loo *et al*, Fernandez *et al* were not able to demonstrate a direct binding of Stx to PMN (48). Furthermore, Holle *et al* could not determine an activation of PMN by Stx *in vitro* (51). This indicates that the question if Stx is bound by PMN does not have a clear answer. This will form part of the research in this thesis.

2.3.2.D Peripheral blood monocytes

The monocyte is another candidate to be the carrier of Stx. Several groups have detected a specific receptor for Stx on monocytes (52, 53, 54). After internalizing, Stx activates the monocytes in contrast to its cytotoxic effect in epithelial cells. This intracellular pathway will be discussed in chapter 2.3.3. In short, the activation of monocytes by Stx consists of initiation of the mitogen activated protein kinase-system (MAPK), nuclear translocation of nuclear factor-κ B, and secretion of pro-inflammatory cytokines like tumor necrosis factor-α and interleukin-6 (52, 55, 56). Since the binding of Stx to monocytes is well-established, it was hypothesized to be the transport-mechanism. The performed experiments on this topic are presented in this thesis.

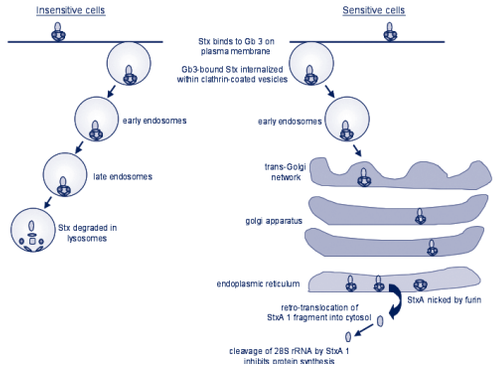
2.3.2.E Other possible carriers

Another possible carrier is a neutralizing factor for Stx2 in human blood, detected by Kimura *et al* (57). After purification, the protein was identified as serum amyloid P (SAP) which is a highly conserved normal human plasma protein and not present in other species. To investigate the function of this binding, Armstrong *et al* determined the amount of circulating SAP in patients with HUS (58). There was no difference in concentration of SAP detected between patients with HUS and control-patients. This indicates that the level of SAP can not predict whether an individual will develop HUS.

2.3.3 Arrival at target cell and intracellular trafficking

The toxin can be internalised after binding to its specific receptor, Gb3. In earlier work, it was described that Stx would enter the cell through a clathrin-dependent pathway. However, clathrin-independent pathways are involved as well (59, 60). Interestingly, not every cell carrying a receptor for Stx is sensitive for the cytotoxic effect of Stx. In monocytes/macrophages, Stx has a stimulating effect. In these cells, which are totally resistant to the cytotoxic function of Stx, the toxin is effectively degraded in lysosomes after having activated the cell (61). However, in toxin-sensitive cells such as Vero cells and cancer cell lines, Stx is able to escape lysosomal degradation and can become cytotoxic after reaching the cytosol (62). In these cells, Stx follows the retrograde transport route. Both intracellular pathways are depicted in figure 2.

Figure 2 Intracellular pathways of Stx



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In retrograde transport, Stx will be able to bypass the late endosome and thereby degradation. After internalizing, Stx is transported from early endosomes to the trans-Golgi network (TGN) and Golgi apparatus (63). This route is controlled by specific proteins (SNARE, Rab) involved in membrane exchange between intracellular compartments (64). After reaching the Golgi apparatus, Stx is efficiently transported to the endoplasmic reticulum (ER) (65). Once Stx arrive in this compartment, it 'misuses' the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol (66). Yu and Haslam demonstrated a complex consisting of Stx and HEDJ. This protein is a chaperone for the transport from the ER to the cytosol (67). When Stx will enter the cytosol, the A-subunit is already cleaved by the enzyme furin. This step is essential for rapid intoxication of the cell (14).

Recent literature on intracellular trafficking has focused on the large variability in the sensitivity for Stx in relation to its targeting to one of the above mentioned pathways. One hypothesis was that there existed a difference in expression of different molecular species of Gb3 on different cell types (15, 68). Interestingly, work of Falguieres *et al*, demonstrated the influence of detergent-resistant membranes (DRM; also called 'lipid rafts') on the trafficking pathway (54). Evidence was provided that association of B-subunits with Gb3-receptors in DRM targeted Stx into retrograde transport. A distinct difference in presence of Gb3-receptors in DRM between Stx-sensitive cells like HeLa-cells and Stx-insensitive cells like monocytes was demonstrated. This could indicate that different molecular species of Gb3 will associate with different areas in the cell membrane, thereby influencing the intracellular pathway of Stx. Another interesting finding concerning the intracellular trafficking is that next to endocytosis, Stx activates Syk and induces rapidly tyrosine phosphorylation of several proteins in Stx-sensitive cells (69). This indicates that next to an inhibitory effect on protein synthesis, Stx is also able to activate sensitive cells.

2.3.4 Effect of Stx on renal non-endothelial cells

Since Stx was detected bound to tubular cells in renal biopsies of HUS-patients, researchers were interested in the effect of Stx on renal cells other than endothelial (16). Work of Hughes *et al* described the cytotoxicity of Stx on glomerular epithelial cells which also express the Gb3-receptor (70). Furthermore, this group demonstrated a stimulation of cytokine production by these cells (71). Recently, Morigi *et al* reported that Stx induced the production of endothelin-1 in murine podocytes, thereby modifying podocyte cytoskeleton and formation of intercellular gaps (72). This damage may occur after the primary insult of the endothelial cells. After perturbation of the endothelial cells and increasing the permeability of the renal filter, Stx can reach the proximal tubular cells. These cells also express Gb3 and urinary markers of proximal tubule damage are elevated ear-ly in the course of HUS (73). Stx is capable of stimulation of these cells to produce cytokines (IL-1, IL-6 and TNF-α).

3 Disorders of complement regulation

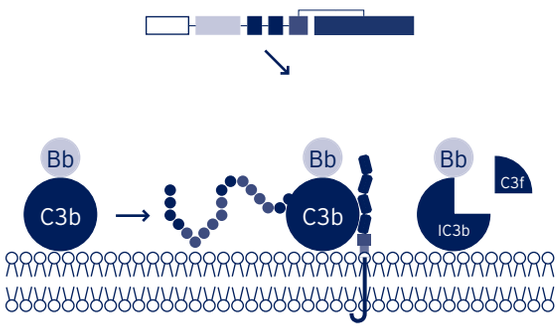
Last few years, research for HUS resulted in an enormous amount of knowledge on non-Stx HUS. This form of HUS does not have a prodromal phase with bloody diarrhoea, but the clinical picture is identical. It can occur both sporadic and in families. The prognosis of non-Stx HUS is considered to be unfavourable with 50% of patients progressing into end-stage renal disease and 25% dying in the acute phase (74).

The extensive search for causes of non-Stx HUS resulted in a paper published by Warwicker *et al* in which they described the segregation of non-Stx HUS to the q32 region of chromosome 1 (75). This chromosome contains a region of complement control proteins called 'regulators of complement activation-locus (RCA)'.

The complement system is part of the innate immunity and is divided in three different pathways according to the initiating element (76). All pathways have the same goal: the formation of the membrane attack complex (MAC; C5b-C9). This complex can induce lysis of bacteria and cells. When a bacteria needs to be eliminated, this is a very

efficient system. But the efficiency of this system depends on a tight control that will prevent non-specific damage to host cells.
The proteins involved in the pathogenesis of non-Stx HUS can be found in the alternative pathway, which is activated by components of the bacterial cell wall. This will lead to formation of C3 convertase which cleaves protein C3 into C3b. Furthermore, in plasma there continuously is a spontaneous hydrolysis of C3 to C3(H20), which is equal to C3b in functionality. C3b is a highly reactive protein. It has two options for binding. When it binds to a bacterium, it will interact with circulating complement proteins called factor B and factor D. This will lead to the formation of the MAC and the bacterium will be destroyed. The other possibility is that C3b will bind to a host cell like an endothelial cell inducing damage with cell lysis, increased expression of adhesion molecules and coagulation factors and the attraction of monocytes and PMN. However, because of a polyanionic coating on the host cell (like sialic acid or heparan sulfate proteoglycans), regulatory complement protein factor H (HF1) will preferentially bind to C3b instead of factor B. Factor H has a higher binding affinity than factor B. Together with other regulatory proteins called Membrane Cofactor Protein (MCP) which is membrane-bound and the circulating factor I (IF), C3b is inactivated. This mechanism is demonstrated in figure 3.

Figure 3 Inactivation of C3b



Regulatory proteins factor H, factor I (both soluble) and MCP (membrane-bound) are involved in the inactivation of highly reactive C3b on host cells.

Since the paper of Warwicker *et al*, mutations in five different proteins involved in complement regulation have been described in patients with non-Stx HUS. These defective proteins will induce an inappropriate regulation of the alternative pathway resulting in excessive damage of host-cells like the endothelium.

3.1 Factor H (HF1)

Figure 4 Factor H

Factor H consists of 20 short consensus repeats. The binding domains for C3b are depicted in light blue. The units that can bind to heparan sulfate or sialic acids are indicated with an arrow.

The gene for HF1 is localized on the chromosome 1. It consists of 23 exons of which exon 10 is cleaved out by alternative splicing. The 22 remaining exons encode the protein which consists of 20 units, called 'short consensus repeats' (SCR) (see figure 4). Exon 1 encodes a leader peptide that is cleaved off. SCR2 is constructed from 2 exons. HF1 is a single-chain serum glycoprotein of 150 kDa (77). HF1 functions as a co-factor in the cleavage of C3b.
More than 50 different mutations in HF1 have been detected in patients with non-Stx HUS (78-85). Most of the mutations cluster in the region of SCR 19 and 20, which is critical for control activation of complement on cell surfaces (77). In non-Stx HUS, HF1 is rarely decreased in plasma (77). Caprioli *et al* showed that in a group of 156 patients with Non-Stx HUS, 30.1% carried a mutation in HF1 (86). These mutated proteins demonstrate defective complement regulation on the endothelial cell (87, 88). Also, anti-HF1 auto-antibodies against its C-terminal recognition function have been described in patients with non-Stx HUS (89, 90).
HF1 has five closely related proteins (complement Factor H-related protein (CFHR)), called CFHR1 to CHFR5. The genes encoding these proteins are also present at chromosome 1. Zipfel *et al* recently described that deletion of CFHR1 or -3, is associated with an increased risk of HUS. They demonstrated with a functional assay that these proteins have a regulatory role in complement activation (91). Interestingly, Jozsi *et al* reported that anti-HF1 auto-antibodies correlated with a deficiency of CFHR1 and -3 (92). Furthermore, a hybrid HF1/CFHR1 gene (exons 1-21 derived from HF1 and exons 22-23 from CFHR1) was detected in a family with HUS by Venables *et al* (93).

3.2 Factor I (IF)

Figure 5 Factor I

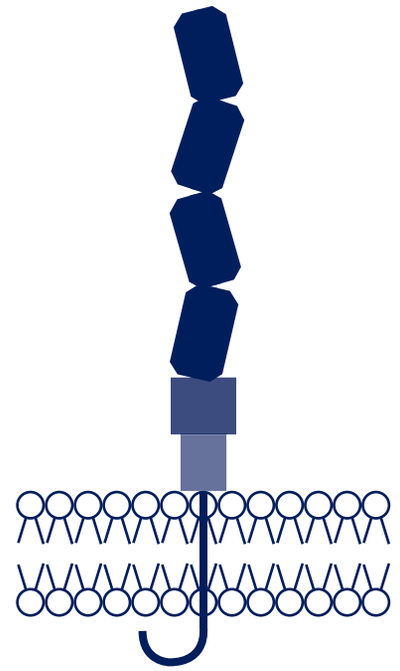
- FI Membrane Attack
- Scavenger Receptor
- Low-density Lipoprotein Receptor
- Unknown function
- Serine protease

Factor I consists of a heavy and light chain, linked by a disulfide bridge

In contrast to the other complement control proteins, the gene for IF is localized on chromosome 4 (position 4q25). It contains 13 exons. The encoded protein consists of a heavy (amino acids 19-335) and a light chain (amino acids 340-583). These two chains are linked by a disulfide bridge (figure 5). In the mature protein, a leader-peptide (amino acids 1-19) and a cleavage-site (amino acids 336-339) are cleaved out. The light chain is the functional serine protease domain which is responsible for the cleavage of C3b and C4b. In this soluble protein, six mutations have been detected in non-Stx HUS patients until now (94, 95, 96). Caprioli *et al* detected a mutation frequency in non-Stx HUS of 4.5% in IF (86).

3.3 Membrane Co-factor Protein (MCP)

Figure 6 Membrane Co-factor Protein



- Complement control protein repeats
- Serine, threonine and proline-rich domain
- Unknown function

Membrane-bound protein MCP consists of 4 complement control protein modules connected by a transmembrane domain and a cytoplasmic tail

MCP is a membrane-bound protein encoded by a gene localized on chromosome 1, position 1q32. The gene consists of 14 exons. The protein is a co-factor for IF and is composed of four complement control protein (CCP) modules that can bind C3b and C4b. CCP 1, 2 and 4 carry an N-bound complex sugar-group. This part is followed by a serine, threonine and proline-rich domain (STP), which is subjected to alternative splicing. This area is O-glycosylated. Then there is a domain with an unknown function, followed by a transmembrane domain and a cytoplasmic tail. The protein structure of MCP can be seen in figure 6. The tail is also alternatively spliced. In combination with the STP-domain, this will lead

to four isoforms that co-exist on the cell-membrane. More than twenty different mutations have been detected in patients with non-Stx HUS (97-100). Most mutations have been detected in the CCP modules. 12.8% of non-Stx HUS patients carry a mutation in MCP (86).

3.4 Other involved complement proteins

All mutations described above result in a decreased function of a complement control protein. This results in an excessive activation of the alternative pathway of the complement system. Recently, Goicoechea de Jorge *et al* described the opposite: gain-of-function mutations in complement factor B (BF) (101). After binding of C3b to a cell surface, BF will bind to it. Then factor D will cleave it into two fragments of which one will form an active serine protease that cleaves additional C3 into C3b (102). The mutated proteins enhanced formation of the serine protease or increased resistance to inactivation by complement regulator proteins, thereby amplifying this pathway of the complement system and resulting in non-Stx HUS (101).
Very recently, another involved complement protein was described by Blom *et al*. They detected in 6 out of 166 HUS patients a polymorphism in C4b-binding protein (103). This protein is also involved in degradation of C3b and functions as a co-factor for IF. The polymorphism decreases the ability of the protein to bind C3b and thereby its degradation.

3.5 Implications of a mutation in complement control proteins

A remarkable feature of the above described mutations is that in the majority of cases it involves a heterozygous mutation. This indicates that the presence of 50% normal protein is inadequate to prevent excessive complement activation. Furthermore, the presence of a mutation is not sufficient to cause non-Stx HUS. There is incomplete penetrance with carriers of a heterozygous mutation who do not develop the disease. Esparza-Gordillo *et al* speculated that concurrence of different susceptibility alleles influences the predisposition to non-Stx HUS (96, 104). Also, unaffected mutation carriers may still develop the disease at an older age in concomitance with pregnancies or infections (86). Thus, it appears that non-Stx HUS is not a simple monogenic disease. Multiple factors (genetic and environmental) are necessary to initiate the pathological process that results in non-Stx HUS. Also, combined mutations in 2 different complement control proteins (HF1 and IF) have been described in patients with non-Stx HUS (105).
The carriage of a mutation in complement control proteins can be considered as a predisposing factor since a trigger like an infection is still necessary to initiate the complement system. The question that still remains is why specifically the renal endothelium is subjected to damage of the dysregulated complement system?

Multiple factors (genetic and environmental) are necessary to initiate the pathological process that results in non-Stx HUS.

The major part of this thesis involves the study of the toxic and damaging effect of Stx on the endothelium, especially the glomerular endothelium.

Chapter 1.2
Aims & outline of the thesis

For many years our research group has contributed to the knowledge of the pathogenesis of HUS. Important findings are the up-regulatory effect of TNF-α on the presence of the Gb3-receptor; the activating effect of Stx on human peripheral blood monocytes; the effect of Stx on its target cell, the renal glomerular endothelial cell; and the binding of Stx to human PMN. In this thesis we have continued our search for a better understanding of the disease mechanism of HUS. In recent years, impressive new insights have been gained in non-Stx HUS. The discovery of the involvement of complement control proteins induced a wave of published articles on this topic. However, our work is still focused on HUS associated with Stx, since the pathogenesis is still unclear after many years of research. But also one article on non-Stx HUS is included. The main questions in our search on HUS remain: (1) how does Stx reach the kidney? (2) Why does Stx specifically damage the kidney? Chapter 2 presents the work on the transport mechanism of Stx in the circulation. After passage of the toxin through the intestinal epithelial cell, it will reach the circulatory system. It is assumed that it will bind to a constituent of the blood, in order to reach its main target: the renal glomerular endothelium. **Chapter 2.1** is focused on the possibilities of PMN as a transporter. Work of te Loo *et al* suggested that PMN are the carriers of Stx in patients with HUS (38). As at a later stage our group and others could not confirm these data, new studies were performed. This chapter describes our efforts to elucidate the interaction of Stx with PMN.

Chapter 2.2 describes the data about the human monocyte as a possible carrier. The monocyte is not sensitive for the cytotoxic effect of Stx, although it carries the specific receptor for Stx. This makes it a good candidate for transport.

The major part of this thesis involves the study of the toxic and damaging effect of Stx on the endothelium, especially the glomerular endothelium. This work is presented in **Chapter 3**. Many experiments have been published on the intracellular trafficking pathway of Stx. A new intracellular pathway, called 'retrograde transport', was demonstrated in Stx-sensitive cells like HeLa cells. **Chapter 3.1** reports about the intracellular trafficking of Stx in its target cells in HUS, the glomerular endothelial cells. Also the mesangial cells were included. This essential knowledge was still lacking.

The Weibel-Palade bodies (WPbs) are an important, endothelium-specific storage organelle. It contains important mediators for inflammation and coagulation. **Chapter 3.2** describes the effect of Stx on these components of endothelial cells. **Chapter 3.3** focuses on the inflammatory process on the endothelial cell induced by Stx. The role of heparan sulfate proteoglycans (HSPG) in the increased adhesion of leukocytes was studied. The last chapter on the interaction between Stx and endothelium, **chapter 3.4**, describes the efforts to understand the dual effect of the toxin. Besides the well-described inhibition of the protein synthesis, Stx can also activate the endothelial cell.

Chapter 4 represents the work on the dysregulation of the complement system in non-Stx HUS. **Chapter 4.1** is a case-report of a non-Stx HUS-patient with a novel mutation in IF. The functional defect of this mutation was demonstrated by performing a functional assay for the alternative pathway of the complement system.

In **chapter 5**, the work of this thesis is summarized and put into perspective in the general discussion. Interesting possibilities for future research are also mentioned.

Chapter 2
Transport of Shiga-like toxin

As the renal microvascular endothelium is the most important target of the toxin, a transport route from the intestine to the kidney is postulated.

Chapter 2.1

Lack of specific binding of shiga-like toxin (verocytotoxin) and non-specific interaction of shiga-like toxin 2 antibody with human poly-morphonuclear leukocytes

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Abstract

Background

After gastrointestinal infection with Shiga-like toxin (Stx) producing *Escherichia coli*, the toxin is transported from the intestine to the renal microvascular endothelium. This is the main target for Stx in humans. Previous studies indicated that polymorphonuclear leucocytes (PMN) could serve as carriers for Stx in the systemic circulation. As at a later stage we could not confirm these data, we performed new studies.

Methods

The binding of Stx1 to PMN was determined *in vitro* (isolated human PMN and whole blood) and *in vivo* (injection in mice). The specificity of binding of an antibody against Stx2 to PMN from patients with haemolytic uraemic syndrome (HUS) was determined. This was compared with binding to PMN from healthy controls, and patients on haemodialysis or on peritoneal dialysis. Furthermore, PMN were incubated with Stx to study possible activation.

Results

No specific binding of Stx1 to PMN could be detected. After intravenous injection of the toxin in mice, it was not associated with PMN. The binding of an antibody against Stx2 to PMN was detected in both patients with HUS and patients after haemodialysis, but not in patients on peritoneal dialysis. Stx was not able to activate PMN.

Conclusions

PMN are not acting as transporter for Stx in the pathogenesis of HUS. The interaction of a Stx-antibody with PMN from HUS-patients is not specific as it can also be observed in patients after haemodialysis (possibly due to activation of the PMN). Therefore, binding of Stx-antibody to PMN is not reliable as a diagnostic tool for HUS.

Introduction

Shiga-like toxin (Stx, also called verocytotoxin) is an important mediator in the pathogenesis of the diarrhoea-associated haemolytic uraemic syndrome (HUS). Stx is produced by the pathogen *Escherichia coli*, of which O157:H7 is the serotype most commonly involved in HUS (1). In an earlier study of 50 isolates of E. coli associated with HUS in The Netherlands, 45 were positive for the Stx2 gene and five for both Stx1 and Stx2 (106).

As the renal microvascular endothelium is the most important target of the toxin, a transport route from the intestine to the kidney is postulated. Stx was never found in sera from patients with HUS, but Stx2 could be detected in kidneys from HUS-patients (19). Furthermore, antibodies against Stx2 are found in serum from patients with HUS (107). After reaching the renal endothelium, Stx will initiate cell damage with the formation of thrombi in the glomerular arterioles and capillaries (1). Acute renal failure with thrombocytopenia and haemolytic anaemia will occur, which are the hallmark features of HUS.

Earlier work of te Loo *et al* suggested that circulating polymorphonuclear leukocytes (PMN) transported Stx2 in patients with HUS (38). However, with a new batch of the same antibody, these results could not be reproduced. Therefore, binding studies with Stx to PMN *in vitro* were repeated. Also, additional binding experiments were performed *in vivo* in mice. The interaction of an antibody against Stx2 with PMN collected from blood of HUS-patients was re-evaluated. It was hypothesized that the antibody could bind aspecifically to activated PMN in these patients. Consequently, the capacity of PMN to bind the antibody against Stx2 was compared between healthy controls, HUS-patients, patients treated by haemodialysis (HD) and peritoneal dialysis (PD). It is known that PMN originating from patients on HD are highly activated (108).

In addition, if no binding of Stx to PMN occurs, it can be proposed that Stx has no effect on PMN function. For this reason, the expression of degranulation markers and cell adhesion molecules (CD66b, CD63, L-selectin and CD11/CD18) on PMN were studied after incubation with Stx2.

Methods

Materials

Purified Stx1 used for the binding experiments was kindly provided by Dr. M.A. Karmali (Public Health Agency of Canada, Ontario, Canada). The 125I-Stx1 B-subunit was a gift from Dr. L. Johannes (Institut Curie, Paris, France). The unlabelled Stx1 B-subunit was a gift from Dr. L. Brunton (University of Toronto, Canada). For the stimulation of PMN, Stx2 was purchased from Toxin Technology (Sarasota, United States). Ethylenediaminetetraacetic acid (EDTA) tubes were ordered from BD Vacutainer (Alphen a/d Rijn, The Netherlands). Ficoll-PaqueTM PLUS was purchased from Amersham Biosciences (Uppsala, Sweden). Human Serum Albumin (HSA; Cealb) from Sanquin (Amsterdam, The Netherlands) was used. Hanks Balanced Salt Solution (HBSS) was ordered from MP Biomedicals (Eschwege, Germany).

Monoclonal antibodies against CD66b (FITC-labelled) and CD63 (PE-labelled) were obtained from Coulter/Immunotech (Marseille, France). Antibodies against L-selectin, CD11b/CD18 (both FITC-labelled), CD13 (PE-labelled) were purchased from DAKO (Heverlee, Belgium), as was the secondary goat-anti-mouse FITC and goat serum. HBSS without phenol red was obtained from Life Technologies (Paisley, Scotland), luminol and 12-myristate 13-acetate and horseradish peroxidase were purchased from Sigma (St Louis, USA).

The antibody against Stx2 was a kind gift from Dr. A. O'Brien (Uniformed Services University of Health Sciences, Bethesda, USA). This is a well-characterized, humanized antibody as described previously (109). The secondary antibody (goat-anti-human FITC) for this antibody was purchased from Jackson Immunoreseach (Cambridgeshire, UK). The Fc-receptor blocker was ordered from Miltenyi Biotec (Utrecht, The Netherlands). Mouse serum was obtained from the Central Animal Laboratory (Nijmegen, The Netherlands). Lipopolysaccharide (LPS) was collected from Sigma (St Louis, USA).

Isolation of polymorphonuclear leukocytes

Fresh venous blood was drawn and collected in EDTA tubes. Blood was diluted two times with PBS and centrifuged (30 minutes 400 xg at 4˚C) over Ficoll-PaqueTM Plus. After centrifugation, the pel-

let containing PMN and erythrocytes was collected. Erythrocytes were lysed using ammonium chloride. This procedure routinely revealed a PMN population of over 95%.

Binding of shiga-like toxin to isolated human polymorphonuclear leukocytes

After isolation of PMN from healthy donors (n=7), cells were washed with PBS and resuspended in HBSS containing 1% HSA at 0˚C. During three hours, 5 x 10^5 PMN were incubated with 125I-Stx1. Incubations were performed with different concentrations of Stx1 ranging from 0.3 up till 70 nmol/L. Stx1 was radio-iodinated according to the Iodogen method. To determine non-specific binding, the same incubation was carried out in the presence of a 25-fold molar excess of unlabeled Stx1. After the incubation, free 125I-Stx1 was separated from the PMN using Ficoll-Paque PLUS and cells were washed. Cell-associated 125I-Stx1 was determined in a gamma-counter. The binding of 125 I-Stx1 to monocytes showed an adequate Kd value in the Scatchard plot analysis. In the glycolipids extracted from human monocytes, 125I-Stx is bound to Gb3.

Binding of shiga-like toxin in whole blood

EDTA-blood from seven healthy human donors was obtained. Whole blood (2-2.5 ml) was incubated with 125I-Stx1 B-subunit (0.75 µgr, 3 µCi) during 2.5 hour at 4˚C.

In order to up-regulate a possible receptor, blood from four donors was pre-incubated with LPS (1 µg/ml) during 2 or 16 hours at 37˚C, before adding the 125I-Stx1 B-subunit.

To investigate the amount of binding of Stx1 B-subunit to PMN in the circulation, 9 normal SE mice (female, age 10 weeks) were injected intravenously with 125I-Stx1 B-subunit. A total amount of 3.5 µg protein (1 µCi) was administered intravenously through the tail vein. In 5 mice all blood was collected after 2.5 minutes. Two of these mice were co-injected with an excess of unlabelled Stx1 B-subunit (100 µg/mouse), to determine the specificity of the binding. The amount of unlabelled Stx1 B-subunit will saturate the specific Stx1 B-subunit binding sites *in vivo*. In 4 mice blood was collected after 15 minutes. One of these mice received 100 µg unlabelled B-subunit. Blood was collected from the orbital plexus. Blood from both mice and men, was centrifuged over Ficoll-Paque PLUS (30 minutes, 400 xg at 20˚C). The different layers (plasma, interphase, Ficoll-Paque PLUS, and the pellet containing PMN & erythrocytes) were separated. The interphase was centrifuged twice to remove platelets (10 minutes, 200 xg). Supernatants were also collected. From each layer a fraction was counted in a gamma counter for the presence of 125I-Stx1 B-subunit. Subsequently, the fraction containing erythrocytes and PMN was lysed with ammonium chloride and washed. By this means, the presence of 125I-Stx1 B-subunit on the PMN could be measured. The 125I activity in each fraction was calculated.

Analysis of binding antibody shiga-like toxin to polymorphonuclear leukocytes

Blood was collected from healthy controls (n=6), patients with HUS (Stx present in faeces) (n=3), patients on PD (n=3) and patients on HD (n=14). After isolation of PMN, cells were incubated with Fc-receptor blocker (15 µl for 15 minutes) and blocked with 10% goat- and mouse serum. Subsequently, PMN were incubated with the antibody against Stx2 followed by goat-anti human FITC. After the incubations the cells were suspended in paraformaldehyde 0.5% and analyzed with FACS analyzer.

Three other antibodies against Stx2 (2 commercial and 1 non-commercial) reacted in a variable degree with normal PMN.

Stimulation of human polymorphonuclear leukocytes with shiga-like toxin

One hundred µl whole blood from four healthy volunteers was incubated with either LPS 1 µg/ml, Stx2 0.1 nM, Stx2 10 nM alone or LPS in combination with Stx2 (0.1 nM or 10 nM) for 2 h at room temperature. Subsequently, erythrocytes were lysed using ammonium chloride. PMN were identified by flow cytometry (Coulter® Epics® XL-MCL) using their morphological criteria and using CD13-PE as specific marker. Monoclonal antibodies against CD63 and CD66b were used as markers of degranulation. Also monoclonal antibodies against L-selectin and CD11b/CD18 were used to study the expression of adhesion molecules. Antigen expression was measured as mean fluorescence intensity of 5000 cells by flow cytometry. Data acquisition and analysis were performed using XL-2 software (Coulter).

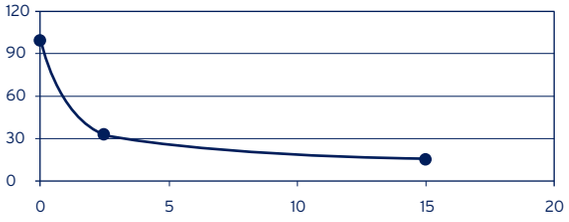
Furthermore, the superoxide production in PMN of eight healthy donors was measured after stimulation with Stx2 0.1 nM, Stx2 10 nM, LPS 1 µM alone or LPS in combination with Stx2 0.1 and 10 nM for 10 minutes, 1 hour and 4 hours, respectively. Phorbol 12-Myristate 13-Acetate (PMA) was used as a positive control. Immediately after the incubation, luminol-enhanced chemilumniscence of proteose-peptone-elicited PMN was measured on a Victor 1420 counter (Wallac, Turku, Finland) at 37˚C. The measurements were performed in 96-well microplates as described previously (110). Each well contained 2 x 10^5 PMN, 50 µM luminal, and 4.5 U/ml horseradish peroxidase in 200 µl of HBSS without phenol red, supplemented with 0.25% HSA. Wilcoxon signed rank test was used to test significance of within-group differences. Differences were considered significant at $P<0.05$.

Results

Binding of shiga-like toxin to isolated polymor-phonuclear leukocytes

PMN from healthy donors were isolated and incubated with 125I-Stx1. To test the specificity of the binding simultaneous incubations were done in the presence of an excess of unlabelled Stx1. In none of the performed experiments (n=7) specific interaction of 125I-Stx1 with PMN was found.

Figure 1 The clearance-curve of shiga-like toxin in mice



Vertical: % I25I-Stx1 B-subunit
Horizontal: minutes

I25I-Stx1 B-subunit was rapidly cleared from the blood of mice. After 5 minutes approximately only 20% was still present.

Binding of shiga-like toxin in whole blood

To establish whether PMN in whole blood can bind Stx, we incubated blood from healthy donors (n=7) with 125I-Stx1 B-subunit. The B-subunit of Stx is the binding part of the toxin, whereas the A-subunit will only stimulate the uptake of the toxin and does not affect binding (111).
The experiment was performed with and without previous stimulation of the blood with LPS. The major part of the B-subunit can be found in the plasma. 71% of total activity was detected in plasma, whereas 0.09% was found in the fraction containing the PMN (n=3). Pre-incubation with LPS did not change the amount of bound toxin (0.09% and 0.02% of total activity after respectively 2 and 16 hours pre-incubation (n=4)).
To investigate the binding of Stx to PMN *in vivo*, 125I-labeled Stx1 B-subunit was injected in mice (n=9). After 2.5 and 15 minutes, blood from mice was collected and the distribution of the B-subunit in the various blood fractions was determined. Stx1 B-subunit rapidly cleared from the blood as is shown in figure 1. Subsequently, the different cell populations were separated and the 125I-Stx1 B-subunit activity in each fraction was determined. The major part of 125I-Stx1 B-subunit was found in plasma. Only a minute fraction of the activity was found in the PMN fraction. The addition of an excess of unlabelled Stx1 B-subunit did not reduce the activity (table 1).

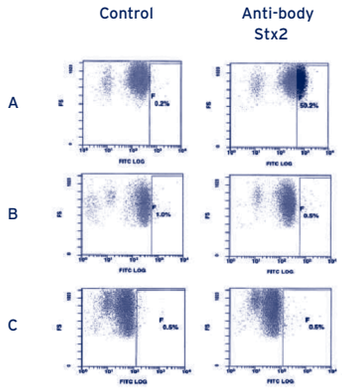
Table 1 Distribution of 125I-Stx1 B-subunit in blood of mice

Time (min)	Unlabelled Stx1-B	Number of mice	Plasma (% of total)	Ficoll (% of total)	Erythrocyte (% of total)	PMN (% of total)	Monocycte + Lymphocyte (% of total)	Platelets (% of total)
2.5	-	3	94.871	3.926	1.568	0.003	0.040	0.373
2.5	+	2	90.566	6.912	2.942	0.006	0.016	0.478
15	-	3	93.302	4.107	1.645	0.006	0.072	0.708
15	+	1	94.504	3.566	1.327	0.003	0.025	0.660

Binding of antibody shiga-like toxin to polymorphonuclear leukocytes

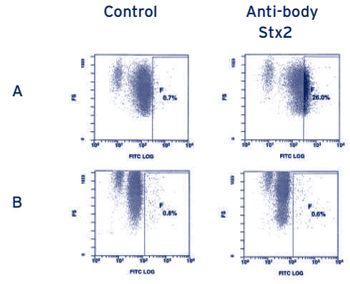
The specificity of attachment of the antibody against Stx2 was tested with PMN of patients with HUS, patients on haemo- or peritoneal dialysis and healthy controls.
In 2 out of 3 blood samples from HUS patients in active disease, the antibody did not show any staining of the PMN (figure 2). Only the PMN of one patient showed an attachment of the antibody to the PMN during active disease, which disappeared during remission.
The healthy controls were negative (n=6). In patients on PD, no attachment of the Stx2 antibody to PMN was found. However, if the antibody was added to PMN from patients on HD, in 11 out of 16 experiments positive staining of the PMN with the antibody was observed (figure 3). In these patients no symptoms of HUS could be detected.

Figure 2 Flow cytometric analysis for detection of antibody against shiga-like toxin 2 on polymorphonuclear leukocytes from patients with haemolytic uraemic syndrome.



PMN of 2 HUS patients were incubated with an antibody against Stx2.
(A) PMN of patient 1 during active disease. (B) PMN of patient 1 during remission. (C) PMN from patient 2 during active HUS.
Binding of the antibody can only be observed in panel A, showing the PMN of patient 1 during active disease.

Figure 3 Flow cytometric analysis for detection of antibody against shiga-like toxin 2 on polymorphonuclear leukocytes of patients on dialysis.



PMN of patients on dialysis were incubated with an antibody against Stx2. (A) PMN from a patient on haemodialysis. (B) This data represents the PMN from a patient on peritoneal dialysis. Binding of the antibody can be observed on PMN originating from patients on haemodialysis. No binding can be found on PMN from patients on peritoneal dialysis.

Stimulation of polymorphonuclear leukocytes with shiga-like toxin

Incubation of PMN with different concentrations Stx2 did not cause any changes in the expression of degranulation markers (CD66b and CD63) on PMN (figure 4). Similar results were obtained with the adhesion molecules. Stx2 did not induce significant changes in the expression of CD11b/CD18 and shedding of L-selectin (figure 4).

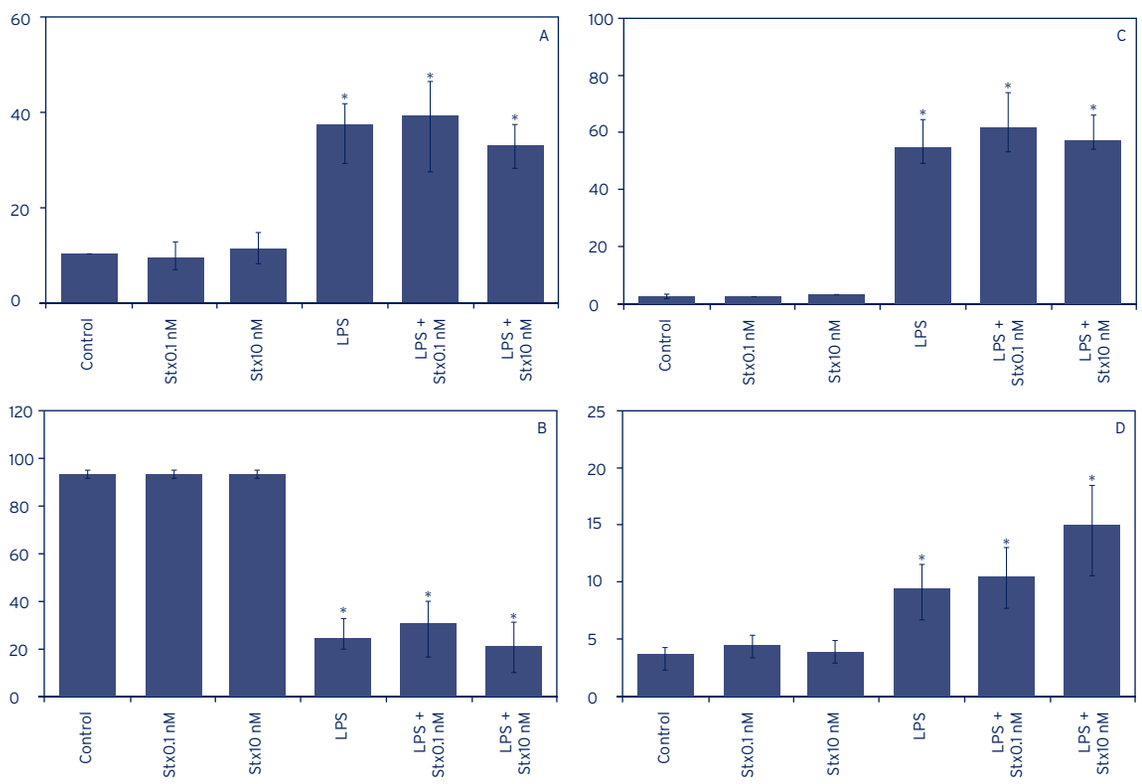
Incubation of PMN from healthy donors did not show an increase of superoxide production whereas LPS induced a small increase (data not shown).

Figure 4 (A, B, C, D) Expression of degranulation markers and cell adhesion molecules on polymorphonuclear leukocytes

Vertical values:

A > % PMN positive for CD11b/CD18 expression
B > % PMN positive for L-selectin expression
C > % PMN positive for CD66b expression
D > % PMN positive for CD63 expression

PMN from healthy donors were stimulated with Stx2 (0.1 or 10 nM) or with LPS alone or in combination with Stx2 (0.1 or 10 nM). After stimulation, the expression of degranulation markers and cell adhesion molecules was determined. (A and B): The expression of cell adhesion molecules (L-selectin and CD11b/CD18 respectively) on PMN is not influenced by incubation with Stx2. (C and D): Also, the degranulation markers (CD66b and CD63 respectively) are not affected by Stx2. However, after incubation with LPS or LPS + Stx2 there is a significant change in expression. P<0.05 is considered to be significant (*).



Discussion

In a previous study, we have demonstrated specific binding of 125I-Stx1 to freshly isolated, non-stimulated human PMN. Also a rapid binding of FITC-labelled Stx1 to PMN was detected when incubated in whole blood (38). In the current study we were unable to reproduce these results. Neither in isolated PMN nor in whole blood a specific binding of Stx1 to PMN was observed. Fernandez *et al* (112) also studied the binding of Stx to PMN. PMN from healthy adults and children were incubated with different concentrations of Stx1 or Stx2 for different time intervals. The binding of Stx to PMN was determined by flow cytometry using specific anti-Stx antibodies, and by quantification of the remnant Stx, evaluating the cytotoxicity of the supernatants on verocells. Neither of the two approaches revealed a positive binding of Stx to PMN surface in any of the conditions assayed. We also evaluated a possible binding of 125I-Stx1 B-subunit after intravenous injection in mice. *In vivo* no specific interaction of Stx1 with PMN could be detected. Some caveats should be noted. It is not allowed to extrapolate data obtained in mice to the human. It is also possible that labelled toxin is detached from cells during the experimental procedure (centrifugation on Ficoll). We have no experience with erythrocytes, but labelled Stx on the surface of monocytes at a constant temperature is not released by centrifugation.

A lack of specific binding of Stx to PMN suggests the absence of a direct effect of Stx on PMN function. Stx2 did not stimulate superoxide production in PMN and was unable to induce the expression of degranulation and activation markers CD63, CD66b and CD11b/CD18. Similar results were reported by Aoki *et al* and Holle *et al* (113, 51).

However, Stx2 also has an inhibitory effect on spontaneous neutrophil apoptosis. Surprisingly, this effect is only partially inhibited by anti Stx2 antibody (114). Could Stx2 interfere with triggering factors for spontaneous apoptosis?

How to reconcile these data with the flow cytometric detection of Stx on PMN from HUS patients by us and recently by Brigotti *et al* (50)? Using flow cytometry, in 13 out of 20 children with HUS they showed binding of anti-Stx mouse monoclonal antibody to PMN. In our last three investigated patients with a proven HUS, PMN from only one patient were clearly positive for the antibody against Stx2 and became negative at remission. A possible explanation is the exposure of altered plasma membrane structures on activated PMN. In D+HUS children, PMN have been found to adhere more avidly to endothelium and induce endothelial injury, assessed morphologically by degradation of endothelial cell fibronectin (45). α1-antitrypsin complexed elastase is raised in HUS-patients reflecting PMN activation and degranulation (46). Fernandez *et al* showed a reduced degranulatory capacity in response to cytokines and a decreased intracellular granule content indicating a preceding process of activation (48). To underpin our hypothesis, we have tested the interaction of the antibody against Stx2 with PMN activated by HD and compared it with PMN obtained from patients treated by chronic peritoneal dialysis. Degranulation of PMN appears to be an ongoing process during HD, starting directly after the initiation (115). In the majority of patients on HD, the activated PMN collected after a HD session showed a binding of the antibody against Stx2. This was not seen in PMN from patients treated by chronic peritoneal dialysis.

From these experiments, we hypothesize that in activated PMN membrane changes take place that cause binding of the Stx2-antibody. The characteristics of these binding sites are still unknown. Therefore, a reliable diagnosis of HUS patients demonstrating the presence of Stx on the surface of PMN by anti-Stx2 is doubtful.

It remains an unsolved issue how Stx is transported in the circulation. Recently, the acute phase protein human serum amyloid P has been suggested by Kimura *et al* as a possible neutralizing factor (57). But if it can also target Stx to the glomerular endothelium needs to be elucidated.

Since a specific treatment for HUS is still lacking, more insight in the transport of this toxin might lead to new intervention strategies.

Chapter 2.2
Interactions of shiga-like toxin with human peripheral blood monocytes

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Abstract

The cytotoxic effect of shiga-like toxin (Stx; produced by certain *Escherichia coli* strains) plays a central role in typical haemolytic uraemic syndrome. It damages the renal endothelium by inhibiting the cellular protein synthesis. Also the monocyte has a specific receptor for Shiga-like toxin, but is not sensitive for the cytotoxic effect. In this work, monocytes are studied as a potential transporter for Stx to the renal endothelium.

Co-incubations of isolated human monocytes loaded with Stx and target cells (vero-cells and human umbilical vascular endothelial cells) were performed. Transfer was determined by measuring the protein synthesis of target cells and by flow cytometry. Furthermore, the effect of a temperature-shift on loaded monocytes was investigated.

Stx loaded monocytes reduced the protein synthesis of target cells. After adding an antibody against Stx, incomplete recovery occurred. Also, adding only the supernatant of co-incubation was followed by inhibition of the protein synthesis. Stx detached from its receptor on the monocyte after a change in temperature and no release was detected without this temperature shift.

Although the monocyte plays an important role in the pathogenesis of HUS, it has no role in the transfer of Stx.

Introduction

The haemolytic uraemic syndrome (HUS) is a clinical syndrome consisting of three characteristic features: haemolytic anemia, thrombocytopenia and acute renal failure (1). In the new classification of HUS, infection due to Shiga-like toxin (Stx) producing bacteria belong to the category of 'etiology advanced' (2). This work is focusing on the form in which Stx producing *Escherichia coli* is the most common pathogen (1). It can produce several types of Stx of which Stx1, Stx2 and Stx2c are most associated with HUS (3, 6). Stx plays a crucial role in the pathogenesis, because of its cytotoxic effect on the renal endothelium. Both renal tubular epithelial cells and glomerular visceral epithelial cells (podocyctes) are also sensitive to the toxic effect of Stx (72, 116). It can inhibit the protein synthesis of these cells after specifically damaging the ribosomal RNA (17). However, the question how this toxin is targeted mainly to the kidney remains unsolved. Stx was never detected in the serum of patients, but it was detected in renal biopsy material of patients with HUS (19). Since a specific treatment for HUS is still lacking, more insight in the transport of this toxin might lead to new intervention strategies.

After oral ingestion of the bacteria through contaminated food or water, the non-invasive bacteria will adhere to the intestinal epithelial cells of the distal small bowel and colon. This will lead to a re-arrangement of the morphology of the cells and initiate inflammation (11, 117). Bacterial flagellin plays an important role in this process (26). Stx can probably reach the circulation because of active transport in these cells and also passively after damage to the intestinal cells (30). Subsequently, it has to be transported in the circulation to reach its primary target, the renal endothelium.

It is very tempting to look at the blood cells as a carrier for the toxin. Stx can bind to a specific receptor which is a globotriaosylceramide (Gb3, Pk Antigen, CD77) (15). This receptor is present on renal endothelial cells, but also on blood cells. Binding of Stx has been described on red blood cells (36), B lymphocytes (118) and platelets which also have an additional binding possibility (glycolipid, band 0.03) (39). Several groups showed the existence of a specific binding of Stx on monocytes (52, 53, 54). After binding to its receptor, Stx can be internalized. Whereas in epithelial cells the toxin follows the retrograde transport route and becomes cytotoxic, in monocytes it is targeted to the lysosomes and will get degraded (54). During this transport, the monocyte becomes activated. This will lead to an increase of transcription factors like NF-κB and AP-1 and an up-regulated production of cytokines like IL-1β, TNF-α, IL-6 and IL-8 (52, 55). These events will have a pro-inflammatory effect.

We postulated that, since the monocyte has a specific receptor, it might also function as a carrier to transport Stx to the renal endothelium. To investigate this hypothesis, Stx was loaded to isolated monocytes from healthy donors and co-incubated with target cells (vero-cells and human umbilical cord venous endothelial cells (HUVECs)). The level of transfer was determined by measuring the protein synthesis of these target cells and by measuring the transfer of FITC-labelled B-subunit of Stx1 with flow cytometry.

Methods

Materials

Stx2 was kindly provided by Dr. M Karmali (Public Health Agency of Canada, Ontario, Canada). Stx1 B-subunit labeled FITC and 125I-Stx1 B-subunit were a gift from Dr. L. Johannes (Institut Curie, Paris, France). Stx1 B-subunit is a useful tool for studying binding in monocytes (53). It is the binding part of the toxin, whereas the enzymatic A-subunit will only stimulate the uptake of the toxin and does not affect binding (111).

Vero-cel medium consists of M199 (Gibco; Paisley/UK), fetal calf serum (FCS, Greiner Bio-One; Krems-munster/Austria), penicillin/streptomycin (Gibco, Paisley/UK) and glutamine (MP Biomedicals; Eschwege/Germany).

HUVECs medium is made of M199, human serum (HS; Cambrex; Walkersville/USA), new born calf serum (NBCS; Gibco, Paisley/UK), penicillin/streptomycin, glutamine, heparine (Leo Pharma BV, Breda/The Netherlands) and endothelial cell growth factor (119). EDTA-tubes were purchased from BD Vacutainer (Alphen aan de Rijn/The Netherlands). The MACS-kit for negative selection of monocytes was provided by Miltenyi Biotec (Bergisch Gladbach/Germany). Hank's balanced salt solution (HBSS) was ordered from MP Biomedicals (Eschwege/Germany). Human serum albumin (HSA) from Sanquin (Amsterdam/The Netherlands) and porcine gelatine from Fluka (Neu-Ulm, Germany) was used. Trichloroacetic acid and TNF-α was obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht/The Netherlands). The antibody against Stx2 (TMA-15) is well-characterised (120). It was a kind gift from Dr. Yamagami from the Department of Biomedical Research from the Teijin Institute, Tokyo, Japan. 3H-leucine and Ficoll-paque PLUS was purchased from Amersham Biosciences (Uppsala, Sweden). Culture plates were ordered from Corning Inc (Corning, USA).

Culture of vero-cells and HUVECs

Vero-cells (renal epithelial cells of the African green monkey) were grown to confluency on a 24-wells plate (ordered from ATCC; Middlesex, UK). These cells have a high basal expression of Stx-receptor CD77.

HUVECs were isolated and these cells were grown to confluence on a gelatine-coated 24-wells plate (121). Every two days fresh medium was added to the cells. In contrast to vero-cells, HUVECs were 24 hours pre-incubated with TNF-α (10 ng/ml) to up-regulate the expression of CD77.

Isolation of monocytes and loading with Stx2

Fresh venous blood (20 ml) from fourty healthy donors was collected into EDTA tubes. Monocytes were isolated by negative selection using antibody-labelled beads (CD3, CD7, CD16, CD56 and CD123). After centrifugation of blood over Ficoll (20 min 400 xg without brake, at room temperature), the interphase (containing monocytes, lymphocytes and platelets) was collected. Platelets were removed by centrifugation (200 xg, 10 min at room temperature) before adding the beads. The purity of monocytes after the magnetic isolation is (as determined by flow cytometry) 80-85%.

After the isolation the monocytes were resuspended in HBSS with 1% HSA and placed on ice. In every experiment, monocytes from one donor were used. To load the monocytes with Stx2, the toxin was added to a concentration of 10 nM during a time period of 3 hr (52). The cells remained on ice. After proper washing to remove all unbound Stx2, the cells were resuspended in vero-cell or HUVECs-medium (in which HS and NBCS are substituted by FCS). Monocytes stayed viable after this loading, as determined by trypan blue exclusion.

Co-incubation Stx2-loaded monocytes and target-cells

These experiments were performed in two different experimental settings; with transfer of loaded monocytes from 4 to 37 degrees, and without change of temperature. To start with the first setting, the Stx2-loaded monocytes were added to a monolayer of target-cells (HUVECs (n=10) or vero-cells (n=9)) in a concentration of $1x10^6$ per well. This was performed during 24 hr at 37˚C. For comparison, also monocytes without Stx were used. To determine the specificity of the effect of Stx2, the loaded monocytes were pre-incubated with a well-characterised antibody against Stx2 (TMA-15, 1 µg/ml, approximately 150 times excess). All experiments were performed in duplicate. To measure the transfer of Stx2 from the monocyte, the protein synthesis of the target-cells was determined by adding 3H-leucine (0.67 µCi/ml). Subsequently, intracellular proteins were precipitated by treatment with TCA and the radioactivity was measured in a liquid scintillation counter.

In the other experimental setting, monocytes were loaded in a similar way with Stx1 B-subunit FITC-labelled. $1x10^6$ loaded monocytes were co-incubated with 1.25×10^5 vero-cells in suspension during 3 hr at 4˚C (n=5), meanwhile continuously rotating. At this temperature, bias due to internalisation of the toxin could be avoided (54, 122). After 3 hr, the presence of Stx1 B-subunit FITC on the vero-cell was determined by flow cytometry. For every experiment at least 1000 cells were measured.

Study of supernatant Stx-loaded monocytes

To study the effect of the supernatant of Stx2-loaded monocytes, it was collected after 16 hrs of co-incubation on vero-cells (n=11). The supernatants were centrifugated to remove possible monocytes and added again to fresh vero-cells. The protein synthesis was measured by adding 3H-leucine and measuring the incorporation after 24 hrs of incubation at 37˚C. Also, the antibody against Stx2 was used to determine a possible effect of free Stx2. Furthermore, monocytes were loaded with 125I-Stx1 B-subunit to investigate whether it could be released after a change in temperature from 4 to 37˚C (n=3). For each experiment, between 3 and $4x10^6$ monocytes are loaded with 200 nM Stx1 B-subunit (3600 cpm/ng protein). After loading, unbound Stx1 B-subunit was removed by centrifugation. Subsequently, the monocytes were placed at 37˚C for 2 hr. Monocytes were centrifugated, and cells and supernatant were measured separately for the presence of 125I-Stx1B with a gamma-counter. This was compared to the amount of binding to the monocytes before the temperature change. The experiment was performed in duplicate.

Statistics

All data represented are expressed as a range with the median. Significance of increase or decrease of protein synthesis compared to controles was analyzed using Wilcoxon Signed Ranks Test. The statistical level of significance was defined as $P<0.05$.

Results

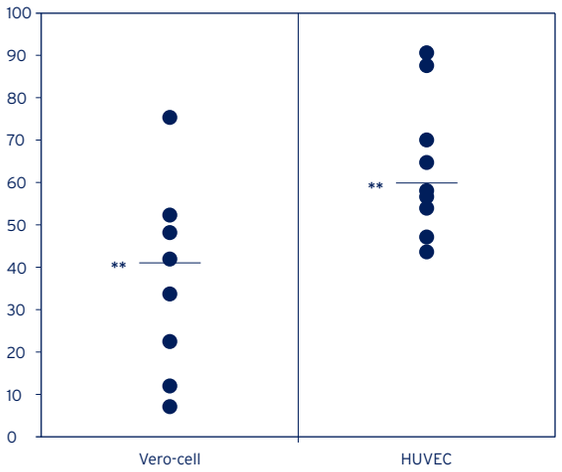
Co-incubation Stx2-loaded monocytes and target-cells

To investigate whether monocytes can transfer Stx2 to target-cells (vero-cell and HUVECs), Stx2-loaded monocytes were added to a monolayer of these cells. After the co-incubations, the protein synthesis of vero-cells and HUVECs was measured. If the Stx2 is transferred to the target-cells, there will be inhibition of the protein synthesis, since this is the biological effect of Stx2 in both types of target-cells.

After 24 hours of co-incubation at 37˚C, in both cell types there is an inhibition of the protein synthesis (figure 1). In vero-cells, more reduction of the protein synthesis can be measured than in HUVECs. Directly adding 10 nM Stx2 to vero-cells reduced the protein synthesis to 5.5% (data not shown). When an antibody against Stx2 was added to the monocytes before co-incubation with vero-cells, the inhibition of protein synthesis is partly re-stored (figure 2). Only in one out of 5 experiments, there was a complete recovery.

In equal experiments performed with HUVECs (n=3), TMA-15 also partly prevented the inhibition of protein synthesis (data not shown).

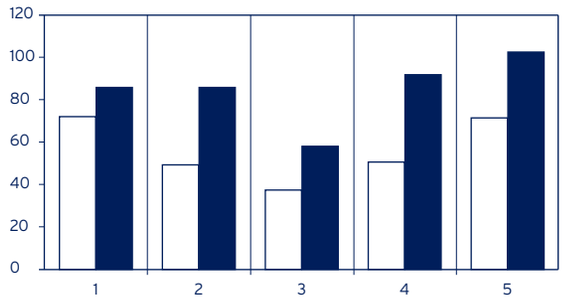
Figure 1 Co-incubation of Stx2 loaded monocytes with target cells (vero-cells and HUVECs)



Vertical: % protein synthesis
****:** P<0.01

To investigate if monocytes can transfer Stx2, toxin-loaded monocytes were co-incubated with vero-cells (n=9) and HUVECs (n=10). This leads to an inhibition of the protein synthesis as can be determined with the incorporation of 3H-leucine. The co-incubation with unloaded monocytes is used as a control and set at 100%.

Figure 2 Effect of the addition of an antibody against Stx2



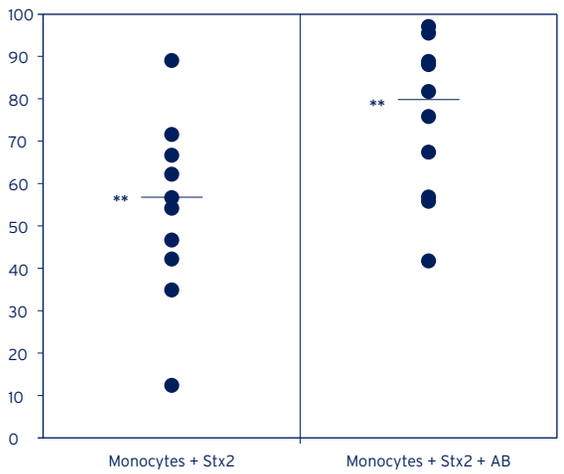
Vertical: % protein synthesis
Horizontal: Donors

The monocytes of 5 healthy donors were co-incubated with vero-cells (Stx2-loaded monocytes, white-bar; Stx2-loaded monocytes with Stx2 antibody, dark blue. The addition of the antibody against Stx2 leads to partial recovery of protein synthesis in 4 donors and complete recovery in one donor. The co-incubation with unloaded monocytes is used as a control and set at 100%.

Study of supernatant Stx-loaded monocytes

Because the addition of an antibody against Stx2 was not sufficient for complete recovery of protein synthesis in four out of 5 tested donors, we investigated the possible presence of an additional inhibitor in the supernatant after 16 hours of co-incubation. This was performed by re-adding the supernatant of co-incubated Stx2-loaded monocytes and a monolayer of vero-cells, to new vero-cells. Figure 3 shows that this incubation again leads to an inhibitory effect on the protein synthesis of the vero-cells, in contrast to the supernatant of unloaded monocytes. But this reduction is less than with direct co-incubation. The effect can be partly blocked by adding the antibody against Stx2. This means that there is unbound Stx2 present in the supernatant. Next, we investigated if the temperature change of the Stx-loaded monocytes from 4 to 37˚C could lead to release of the toxin. For this reason, we loaded the monocytes with 125I-Stx1 B-subunit. The amount of radio-activity on or inside the monocytes was measured before and after the incubation of the cells for 2 hr at 37˚C (table 1). Vero-cells were used as a control. Both in vero-cells as in monocytes, the toxin is released from its receptor after the incubation.

Figure 3 Addition of supernatants co-incubation to vero-cells



Vertical: % protein synthesis
****:** P<0.01

After co-incubations of Stx-loaded and unloaded monocytes, supernatants were collected and re-added to fresh vero-cells (n=11). The supernatant from the toxin-loaded monocytes induced again an inhibition of the protein synthesis. This inhibition could be partially restored with a Stx2-antibody (AB). The incubation of supernatant with unloaded monocytes was used as a control and set at 100%.

Table I Amount of 125I-Stx1 B-subunit on cells after change of temperature

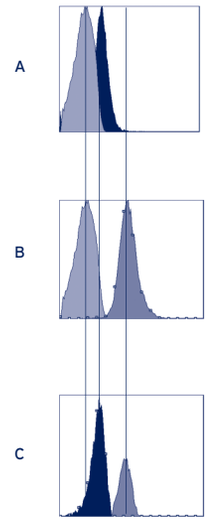
Stx-loaded cells	**Before 4 > 37 C** (cpm)	**After 4 > 37 C** (cpm)
Vero-cells	1034383	671060
Monocytes donor 1	1437	427
Monocytes donor 2	1070	374
Monocytes donor 3	1121	677

cpm= counts per minute

Transfer of Stx to target-cells without change in temperature

A change in temperature from 4 to 37˚C, released Stx from its receptor on the monocyte. To study again the possibility of transfer of Stx from monocyte to target-cells, we loaded isolated monocytes with Stx1 B-subunit labelled with FITC. A co-incubation of these monocytes with vero-cells in suspension without a change in temperature, did not result in a transfer of the B-subunit to the vero-cells (n=5, figure 4).

Figure 4 Transfer Stx-loaded monocyte to vero-cell without temperature shift



Horizontal: intensity FITC
Vertical: amount of cells

(light blue: normal monocytes; dark blue: Stx-loaded monocytes and blue: vero-cells). The x-axis represents the intensity of the FITC-signal, the y-axis represents the amount of cells.
Panel A: Stx1 B-subunit FITC-labelled was bound to monocytes from a healthy donor showing an increase in intensity. B: Co-incubation of vero-cells and unloaded monocytes. Notice the high basal signal of vero-cells.
C: After 2 hr co-incubation with vero-cells in suspension at 4 oC, there is no transfer of the FITC-signal. The cells remain at their position.

Discussion

After co-incubation of Stx2-loaded monocytes with target-cells (vero-cells and HUVECs), an inhibition of the protein synthesis could be detected. Since the biological effect of Stx is the inhibition of protein synthesis, a transfer was expected. This effect can not be due to the presence of monocytes, because it is well-described that Stx does not have an inhibitory effect on peripheral blood monocytes (52). However, the addition of an antibody against Stx could only partly restore the inhibition. We hypothesized that in parallel to transfer of Stx2, also a possible additional inhibiting factor was present in the supernatant during the experiment. To further investigate this possibility, the supernatant of this co-incubation was re-added to fresh vero-cells. Again there was an inhibition of the protein synthesis, which could partly be decreased by blocking with the Stx2-antibody. The conclusion was made that there is indeed another inhibitory factor present (possibly released by activated monocytes), but also unbound Stx2 was present (123).

Apparently, Stx1 B-subunit is released from its receptor when there is shift of temperature from 4 to 37˚C as shown in this paper and also by Ramegowda & Tesh (53). This finding underlines the caution that has to be taken, in drawing conclusions from *in vitro* experiments performed at 4˚C. In figure 5 all findings are schematically summarized.

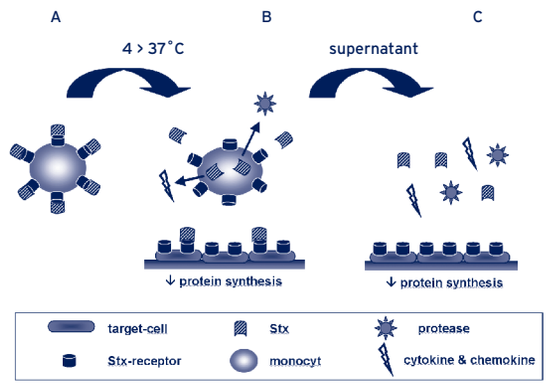
Since these *in vitro* experiments are not suitable to investigate a possible transfer, experiments were performed without a change of temperature. No transfer of the binding part of the toxin could be detected in this setting. Experiments can not be performed at 37˚C because the toxin will be internalised by the monocyte during 3 hours of incubation.

Because of these *in vitro* experiments, we believe that the monocyte can not function as a transporter for Stx in the circulation. However, monocytes still seem to play an important role in the pathogenesis. As a component of the innate immune system, they play a central role in immunity and inflammation. Fernandez *et al* showed that patients in the acute period of HUS have monocytes with phenotypic (reduced expression of CD14, CD64 and CD11b) and functional differences (decreases LPS-induced TNF-α production and Fcγ-dependent cytotoxicity) compared to healthy children and acute uremic children (124).

Some unanswered questions remain. What is the inhibitory factor released by monocytes after loading with Stx? And why is there still unbound Stx2 present in the supernatant after 16 hours of co-incubation? It is surprising that Stx2 is not completely bound and internalised by the numerous receptors on the monocytes and the vero-cells. And also the question how Stx is transported in the circulation remains unsolved. Kimura *et al* described that serum amyloid P (SAP) can bind Stx2 and function as a neutralizing factor (57). However, in humans there was no correlation between circulating SAP and the development of HUS (58).

Since renal endothelial damage is already present after the occurrence of clinical symptoms of HUS, it is very important to develop efficacious early prevention. Understanding the mechanism in which the toxin is specifically targeted to the kidney, can lead to novel intervention strategies.

Figure 5 Schematic summary of performed experiments



A: Monocytes loaded with Stx at 4˚C.
B: After shifting Stx-loaded monoytes to target-cells at 37˚C, Stx will be released from its receptor. This will lead to inhibition of protein synthesis of target cells. But, transport can not be excluded (but this was performed in experiments without a shift in temperature). Probably, some toxin will be internalised and monocytes become actived. This can lead to production of cytokines and proteases (possible cytotoxic factors).
C: When the supernatant is re-added to new target-cells, there are released Stx and secreted products present. This will induce again an inhibition of the protein synthesis.

Chapter 3
Effect of Shiga-like toxin on endothelium

The transport route of Shiga-toxin/Stx-1 was never investigated in primary renal cells, the main target for Stx-1 in humans.

Chapter 3.1
Trafficking of Shiga Toxin/Shiga-like toxin-1 in human glomerular microvascular endothelial cells and human mesangial cells

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Abstract

This study has determined the intracellular transport route of Shiga-like toxin (Stx) and the highly related Shiga toxin in human glomerular microvascular endothelial cells (GMVECs) and mesangial cells. In addition, the effect of tumor necrosis factor-α (TNF-α), which contributes to the pathogenesis of haemolytic-uraemic syndrome, was evaluated more profound. Establishing the transport route will provide better understanding of the cytotoxic effect of Stx on renal cells. For our studies, we used receptor-binding B-subunit (StxB), which is identical between Shiga toxin and Stx1. The transport route of StxB was studied by immunofluorescence microscopy and biochemical assays that allow quantitative analysis of retrograde transport from plasma membrane to the Golgi apparatus and the endoplasmic reticulum (ER). In both cell types, StxB was detergent-resistant membrane associated and followed the retrograde route. TNF-α up-regulated Gb3 expression in mesangial cells and GMVECs, without affecting the efficiency of StxB transport to the ER. In conclusion, our study shows that in human GMVECs and mesangial cells, StxB follows the retrograde route to the Golgi apparatus and the ER. TNF-α treatment increases the amount of cell-associated StxB, but not retrograde transport as such, making it likely that the strong TNF-α-induced sensitization of mesangial cells and GMVECs for the toxic action of Stx is not due to a direct effect on the intracellular trafficking of the toxin.

Introduction

Shiga-like toxins (Stx) play a key role in the pathogenesis of the diarrhoea-associated haemolytic-uraemic syndrome (HUS). Stx, produced by bacteria like *Escherichia coli* and *Citrobacter freundii*, are exotoxins that have damaging effects on the endothelial cells in the glomeruli and arterioles of the kidney (11). This injury leads to cell death and the initiation of the coagulation cascade. Consequently, the three classical features of HUS will occur: acute renal failure, thrombocytopenia, and haemolytic anemia. It is not yet known why Stx have a specific effect on the renal endothelium.

Stx are highly similar to Shiga toxin from *Shigella dysenteriae*. These toxins are all composed of a catalytic A-subunit, and a receptor-binding homopentameric B-subunit (StxB) that can bind to a specific functional receptor. The A-subunits of Stx-1 and Shiga toxin differ in only one amino acid, whereas the amino acid sequences of the respective B-subunits are totally identical. In the following, both toxins will be considered as equivalent molecular entities. The receptor of Shiga toxin/Stx-1 is a glycosphingolipid, called globotriaosylceramide, Gb3 or CD77 (125). After binding to this receptor, it is suggested that Shiga toxin/Stx-1 can enter cells via both clathrin-dependent and –independent endocytosis (59, 68, 126, 127).

Shiga toxin/Stx-1 induces different biological effects in various human cell types on which its functional receptor is present. In monocytes/macrophages, Shiga toxin/Stx-1 has a stimulating effect that consists of activation of the mitogen activated protein kinase-system (MAPK), nuclear translocation of nuclear factor-κ B, and secretion of cytokines like tumor necrosis factor-α (TNF-α and interleukin-6 (52, 56). In these cells that are totally resistant to the cytotoxic function of Shiga toxin/Stx-1, the toxin is effectively degraded in lysosomes after having activated the cell (54). This transport route can also be demonstrated in bovine intestinal epithelial cells (128). However, in toxin-sensitive cells such as cancer cell lines, Shiga toxin/Stx-1 is able to escape lysosomal degradation and can become cytotoxic (62). In these cells, Shiga toxin/Stx-1 follows a recently described pathway, termed the retrograde transport route.

Retrograde transport permits Shiga toxin/Stx-1 to bypass the late endocytic pathway by trafficking directly from early/recycling endosomes to the trans-Golgi-network (TGN) (63). This step is dependent on Rab6a', SNARE complexes around the heavy chain soluble N-ethymaleimide-sensitive factor attachment proteins (SNAREs), syntaxin 16 and -5 (64, 129), the tethering molecule golgin97 (130), dynamin, clathrin (126, 127), and the potential clathrin adaptor epsinR (127). A correlation has also been described between the retrograde trafficking of Shiga toxin/Stx-1 at the early/recycling endosomes-TGN interface and its association with detergent-resistant membranes (DRMs). When Shiga toxin/Stx-1 is found in the DRM fractions, for example in HeLa cells, the protein is targeted into the retrograde route. In monocytes/macrophages, Shiga toxin/Stx-1 is not associated with DRMs, and retrograde transport is inefficient (54). From the TGN, Shiga toxin/Stx-1 is transported to the endoplasmic reticulum (ER) using a KDEL-receptor and coatomer protein complex I (COPI)-independent pathway (131). After reaching the ER, Shiga toxin/Stx-1 most likely uses the cellular retrotranslocation machinery to transfer the catalytic A-subunit to the cytosol (66, 67).

The A-subunit functions as RNA N-glysosidase. In the cytosol, it removes one adenine from the 28 -S rRNA of the 60S ribosomal unit and thereby inhibits the elongation factor 1–dependent aminoacyl-tRNA binding to ribosomes (17, 18). This will lead to an inhibition of peptide elongation and consequently an overall inhibition of protein synthesis.

The transport route of Shiga toxin/Stx-1 was never investigated in primary renal cells, the main target for Stx-1 in humans. In this study, we used quantitative StxB-based trafficking tools and immunofluorescence to analyze whether Shiga toxin/Stx-1 was targeted to the retrograde route in human glomerular and mesangial cells. Both cell types are known to express Gb3, which can be up-regulated by cytokines like TNF-α (132, 133, 134). This cytokine can be found elevated in serum of HUS-patients (135). Also, Harel *et al* found a kidney-specific induction of the TNF-α synthesis after the injection of mice with Stx-1 (136). Therefore, we have studied the influence of TNF-α on the trafficking of StxB. As expected, we found that TNF-α increases Gb3 expression and thus, the quantity of cell-associated toxin. However,

TNF-α does not affect StxB association with DRMs and the efficiency of retrograde StxB transport to the ER.

Materials & Methods

Cells and cell culture

Isolation of glomeruli, as well as purification, characterization and cell culture of human GMVECs and human mesangial cells was performed as described by Van Setten *et al* (132, 133). HeLa cells were cultured as previously described by Johannes *et al* (137). Cells were used for maximal 12 passages. All experiments in this manuscript were performed at least three times on human GMVECs or mesangial cells obtained from at least two different donors. The cells were preincubated with a 100 ng/mL dose of TNF-α (Roche, Basel, Suisse). This dose effectively induced expression of Gb3 on GMVECs without affecting the viability of the cells (132). The human monocytes were obtained from blood of healthy donors with their prior consent according to published procedures by Faradji *et al* (138). Monocytes were differentiated into macrophages using CSF-1, as described by Falguieres *et al* (54).

Glycolipid extraction and thin-layer chromatography (TLC)

Lipid extraction was performed as described by Falguières *et al* (54) according to the method of Bligh and Dyer (139). The isolated glycolypids were spotted on high-performance thin-layer chromatography (HPTLC) plates (Science Inc., Agton Hights, IL, USA) and separated with chloroform: methanol: water (65:25:4). The plates were dried and incubated with STxB, polyclonal anti-STxB (obtained as previously described (54)), and alkaline phosphatase coupled antibodies (Jackson Immunoresearch, West Grove, PA, USA). Reactive bands were revealed with the use of ECF (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and quantified with a PhosphorImager (GE Healthcare, Buckinghamshire, UK) using a standard Gb3 preparation as reference.

Immunofluorescence methods

Human GMVECs and mesangial cells were grown on gelatine-coated glass coverslips and preincubated or not with TNF-α (100 ng/mL) for 24 hr. The cells were placed on ice and incubated with Cy3-StxB or Cy3-StxB-Glyc-KDEL (obtained as previously described, (54)) for 30 minutes. Cy3 labeling of StxB was done according to the manufacturer's instructions (GE Healthcare). After washing with cold (4˚C) culture medium, the cells were incubated with warm (37˚C) complete culture medium (containing 20% fetal calf serum (FCS) instead of 10% new born calf serum (NBCS) and 10% human serum (HS)) for 4 or 24 hr at 37˚C. Cells then were washed with phosphatebuffered saline, fixed at room temperature for 15 min in 4% paraformaldehyde, quenched with ammonium chloride, permeabilized with 1% saponine, incubated with the indicated primary (the antibody against Golgi-marker CTR433 was a kind gift from Dr. M Bornens, Institut Curie, France; calnexin-anti-body is purchased from BD Transduction Laboratories, Erebodegem, Belgium) and secondary anti-bodies (anti-mouse FITC; purchased from Jackson Immunoresearch, West Grove, USA), mounted, and viewed by confocal microscopy (Leica Microsystems, Mannheim, Germany).

Iodination and detergent resistant membrane isolation

According to the manufacturer's instructions for iodination, 150 µg of purified StxB-Glyc-KDEL was treated with 1 mCi iodine-125 (>15 Ci/mg, GE Healthcare) on a single IODO-BEAD (Pierce Biotechnology, Rockford, IL, USA). Non-incorporated iodine was removed on PD10 gel filtration columns (GE Healthcare). StxB-Glyc-KDEL was labeled to a specific activity of about 5000 cpm/ng. DRMs were isolated on Optiprep (Sigma-Aldrich, St Louis, MO, USA) gradients, as published (54). The DRMs were found at the 5/30% interface.

Glycosylation assay

To obtain quantitative information on retrograde transport of StxB to the ER, a modified version of StxB, StxB-Glyc-KDEL, was used (purified as published, (54,137)). Confluent GMVECs, mesangial cells and HeLa cells were preincubated with or without TNF-α (100 ng/mL) for 24 hours. Binding of [125I]-labeled StxB-Glyc-KDEL (50 nM) was performed for 30 minutes on ice in complete medium containing 20% fetal calf serum. After incubation at 37˚C for the indicated periods of time in the same culture medium, cells were washed with phosphate-buffered-saline and lysed in SDS sample buffer (187,5 mM Tris/HCl pH 6.8, 6% SDS, 30% glycerol, 0,03% Phenol Red). Lysates were analyzed by 10-20% gradient SDS-PAGE gels, and autoradiographs were quantified by PhosphorImager analysis (GE Healthcare) using ImageQuant software (GE Healthcare). The percentage of glycosylated over total cell-associated StxB-Glyc-KDEL was determined.

Sulfation assay

A variant of StxB, StxB-Sulf2, was used to obtain quantitative information on the passage of StxB into the Golgi apparatus (purified as published (54, 63)). Briefly, after 24 hr of preincubation with or without TNF-α (100 ng/ml), GMVECs, mesangial cells, and HeLa cells were sulfate starved for 90 minutes. After binding of StxB-Sulf2 to cells for 30 minutes on ice, the sulfate starved cells were incubated for 4 hr at 37˚C in $35SO_4^{2-}$ containing medium (400 µCi/ml). The cells were lysed in lysis buffer (1% Triton in phosphate-buffered saline containing Protein Inhibitor Cocktail), and a post nuclear supernatant was prepared. StxB-Sulf2 was immunoprecipitated with monoclonal anti-StxB antibody 13C4 (obtained as previously described, (54)) and protein-G agarose beads. Washed precipitates were boiled in SDS sample buffer, loaded on Tris-Trycine gels and analyzed by autoradiography. Sulfation of StxB-Sulf2 was quantified by PhosphorImager analysis (GE Healthcare) using ImageQuant software (GE Healthcare). Global sulfation of endogenous proteins and proteoglycans was determined by trichloracetic acid precipitation of the immunoprecipitation supernatant. The precipitate was retained on GF/C glass fiber filters (Whatmann, Maidstone, United Kingdom) and radioactivity was counted in a liquid scintillation counter.

Results

Gb3 extraction

To confirm the presence of Gb3 on human GMVECs and mesangial cells, neutral glycolipids were extracted from unstimulated or TNF-α stimulated cells, separated by thin-layer chromatography, and Gb3 was specifically detected using an overlay technique (9, 22, 23). The amount of Gb3 was quantified by PhosphorImager (table 1). As expected, GMVECs and mesangial cells expressed Gb3. Expression levels in unstimulated mesangial cells were comparable to HeLa cells, and about two fold higher than those found in unstimulated GMVECs. Pretreatment with TNF-α resulted in a two-fold increase of Gb3 levels in mesangial cells, and a five-fold increase in GMVECs. Thus, after treatment with TNF-α, GMVECs and mesangial cells expressed similar Gb3 levels.

Transport to the Golgi apparatus

In HeLa cells, StxB traffics to the Golgi apparatus by retrograde transport (54). To determine if StxB has access to the retrograde route in human GMVECs and mesangial cells, immunofluorescence experiments were performed with or without pretreatment of the cells with TNF-α. After binding and 4 or 24 hr of internalization of Cy3-labeled StxB or StxB-Glyc-KDEL, respectively, the cells were fixed, quenched, permeabilized, and incubated with an antibody against the Golgi marker CTR433. The intracellular distribution of StxB was similar in TNF-α stimulated and unstimulated cells, and in the following, only the immunofluorescence data on unstimulated cells are shown.

After 4 hr of internalization of Cy3-STxB, a significant overlap between the Golgi-specific marker CTR433 (green) and the StxB (red) was observed in GMVECs and mesangial cells (figure 1), strongly suggesting that after 4 hr, StxB was targeted to the Golgi apparatus. Immunofluorescence experiments were also performed after 24 h of internalization. For this condition, a KDEL-tagged variant of StxB was used. The KDEL peptide does not increase the efficiency of StxB transport to the ER, but rather improves the ER retention of the protein (137). Consistently, other studies showed that interfering with KDEL-receptor function did not affect retrograde transport of Stx to the ER (140). After 24 hr of internalization, no more overlap between the Golgi-marker CTR433 (green) and Cy3-StxB-Glyc-KDEL (red) was observed in GMVECs or mesangial cells. The observed staining pattern only partially overlapped with the ER marker calnexin (not shown), and the exact identity of the compartment in which StxB accumulated after 24 hours remains to be identified.

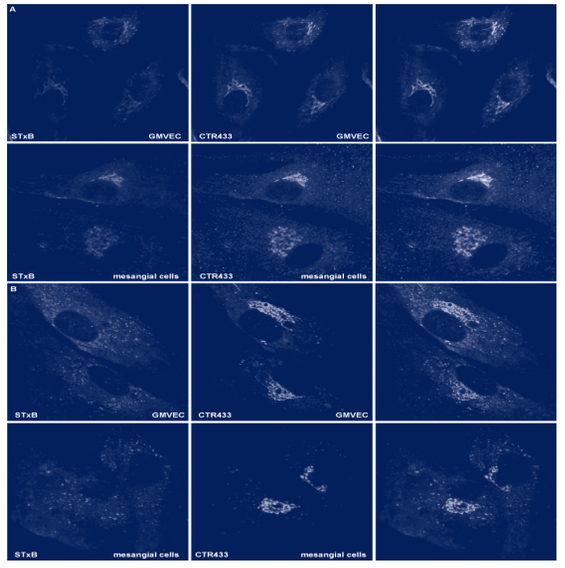
To confirm that StxB is transported to the Golgi apparatus, a biochemical population-based approach was used. The StxB-Sulf2 variant contains a tandem of protein sulfation sites that are recognized by TGN-localized sulfotransferase, which catalyzes the post-translational transfer of sulfate from the medium onto these sites (54). After binding on ice and internalization at 37˚C of StxB-Sulf2 for 4 hr in [35S] sulfate containing medium, StxB-Sulf2 was immunoprecipitated and sulfated protein was analyzed by autoradiography. Radiolabeled StxB-Sulf2 could be detected in GMVECs and mesangial cells (figure 2). HeLa cells were used as positive control and human monocyte derived macrophages, in which retrograde transport cannot be detected, as a negative control (54). Stimulation of GMVECs and mesangial cells by TNF-α increased the sulfation signal, reflecting the increased Gb3 expression under these conditions (see table 1).

Table I Quantification of Gb3 expression

Cell types	Gb3 (μg/10^6 cells)
HeLa cells	0.94
Mesangial cells	1.15
Mesangial cells + TNF-α	2.00
GMVECs	0.50
GMVECs + TNF-α	2.90

After extraction of neutral glycolipids, Gb3 was detected by overlay and quantified on TLC plates. Standards were run in parallel.

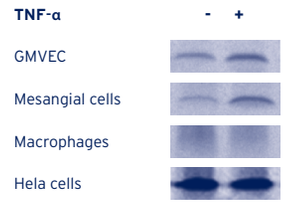
Figure I* Immunofluorescence analysis of StxB transport to the Golgi apparatus in human mesangial cells and GMVECs



*** To see figure in full colour, see Appendix**

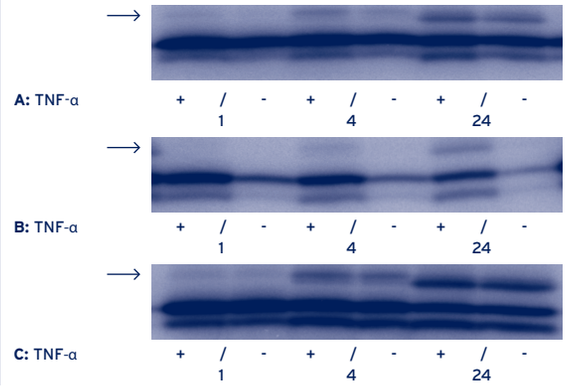
A. Cy3 labeled StxB (red) was internalized for 4 hours in human GMVECs or in human mesangial cells. Cells were fixed and stained for the Golgi marker CTR433 (green). StxB and CTR433 colocalize (yellow) in both human GMVECs and human mesangial cells. B. Cy3 labeled StxB-Glyc-KDEL (red) was internalized for 24 hours, and then fixed and stained for the Golgi marker CTR433 in human GMVECs or mesangial cells. No more accumulation of StxB in the Golgi region is observed. Similar figures were obtained after 24 hr incubation with Cy3 labeled StxB (data not shown).

Figure 2 Sulfation analysis of StxB transport to Golgi apparatus in human GMVECs and human mesangial cells



Cells were preincubated with or without TNF-α. After binding StxB-Sulf2 was internalized into GMVECs, mesangial cells, macrophages and HeLa cells in the presence of radioactive sulfate. Then cells were lysed, StxB was immunoprecipitated and analyzed by gel electrophoresis. Radiolabeled StxB-Sulf2 was revealed by autoradiography. Sulfated, i.e. TGN associated StxB-Sulf2 was detected in HeLa cells, GMVECs and mesangial cells.

Figure 3 Glycosylation analysis of StxB transport to the ER



1, 4 & 24: Incubation time (hr)

Cells were incubated with or without TNF-α. After binding of iodinated StxB-Glyc-KDEL on ice, the protein was internalized into human mesangial cells (A), human GMVECs (B) and HeLa cells (C) for 1, 4 or 24 hours. Then cells were lysed and lysates were analyzed by gel electrophoresis. The arrow indicates the glycosylation product, i.e., ER associated StxB. The middle band is non-glycosylated Shiga toxin. The lower bands are proteolytic cleavage products.

Transport to the ER

In HeLa cells, StxB reaches the ER by retrograde transport (54, 63, 137). To determine if StxB is also transported to the ER in human GMVECs and mesangial cells, a modified version of StxB, StxB-Glyc-KDEL, was used. StxB-Glyc-KDEL contains a N-glycosylation site at the C-terminus, which is glycosylated by ER-localized oligosaccharyl transferase, allowing to monitor and quantify arrival in this compartment (54, 137). As discussed above, the KDEL tag ensures efficient retention in the ER. Iodinated StxB-Glyc-KDEL was bound to and internalized into GMVECs, mesangial cells, and HeLa cells as a positive control. After 1, 4, or 24 hr, glycosylated StxB-Glyc-KDEL was detected and quantified by autoradiography after SDS-PAGE as the percentage of total cell associated StxB-Glyc-KDEL. It is important to indicate that this assay system measures the glycosylation efficiency independently of variations in receptor numbers, and thus, allows comparison of the glycosylation efficiency – reflecting retrograde transport to the ER – between different experimental conditions.

Figure 3 shows representative gels from all 3 cell types that were analyzed after preincubation or not with TNF-α. The uppermost band, indicated by an arrow, represents the glycosylation product, and the lowermost band is a proteolytic cleavage product. All bands are visible in all conditions. Quantification of the glycosylation bands is shown in table 2. In HeLa cells, glycosylation increases from 1.1% over 6.4% to 24.9% after respectively, 1, 4, or 24 hr. These figures reproduce previously reported values (137), and reflect the initial observation that glycosylation of StxB-Glyc-KDEL is slower than its morphologically detected arrival in the ER ((137): immunofluorescence studies; (141): electron microscopy studies). This is likely due to inefficient recognition by oligosaccharyl transferase of folded StxB-Glyc-KDEL and possibly also to partial targeting of StxB-Glyc-KDEL to the smooth ER (142). In mesangial cells, glycosylation efficiency is systematically lower than in Hela cells, even at the earliest time points. This difference becomes particularly striking after 24 hr (table 2). In GMVECs, the glycosylation efficiency is comparable to Hela at the earlier time points, even though some decrease is already apparent after 4 hr. After 24 hr, the glycosylation values observed on GMVECs are almost as low as in mesangial cells (table 2). Separated experiments showed lack of glycosylation of StxB-Glyc-KDEL in human monocyte-derived macrophages.

Strikingly, 24 hr stimulation with TNF-α did not increase retrograde transport efficiency in any of the analyzed cell types at any of the analyzed time points (table 2).

Table 2 Quantification of STxB retrograde transport to the ER.

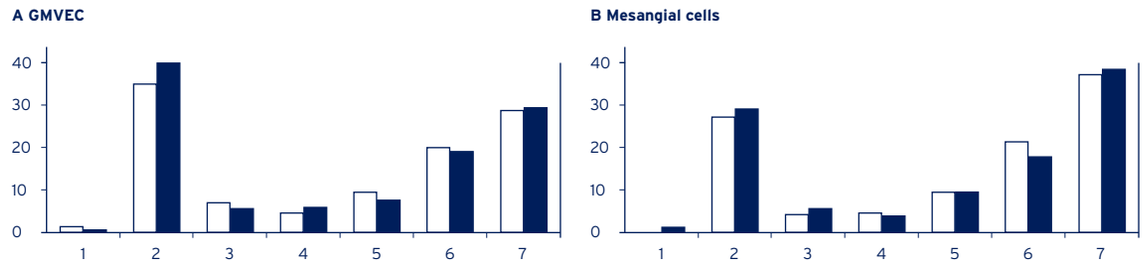
Incubation time	Cell type	Glycosylation (%)	
		Control	TNF-α
1 hour	GMVECs	1.6 ± 0.9 (4)	1.5 ± 0.6 (2)
	mesangial cells	0.6 ± 0.1 (3)	0.5 ± 0.2 (2)
	HeLa cells	1.1 ± 0.8 (3)	0.6 ± 0.1 (2)
4 hours	GMVECs	5.5 ± 3.5 (3)	5.0 ± 1.1 (3)
	mesangial cells	3.8 ± 3.3 (3)	1.9 ± 0.6 (3)
	HeLa cells	6.4 ± 1.5 (4)	6.9 ± 1.0 (3)
24 hours	GMVECs	9.2 ± 2.8 (6)	10.3 ± 3.0 (4)
	mesangial cells	7.4 ± 1.2 (5)	7.2 ± 1.7 (5)
	HeLa cells	24.9 ± 1.6 (6)	23.7 ± 1.9 (5)

After 24 hours of preincubation with or without TNF-α , iodinated STxB-Glyc-KDEL was bound to GMVECs, mesangial cells and HeLa cells for 1, 4, or 24 hours. Then cells were lysed and lysates were analyzed by gel electrophoresis. Arrival in the ER was quantified through the detection of STxB's glycosylation. Data given as means ± SEM (number of experiments in parentheses).

DRM association

It has previously been shown that efficient retrograde transport of StxB correlates with efficient DRM association (54). To determine whether in mesangial cells and GMVECs, StxB is also associated with DRMs, iodinated StxB-Glyc-KDEL was bound to TNF-α treated or control cells on ice. Cells were lysed in Triton X-100 buffer, DRMs were floated on gradients, which were fractionated. DRMs were found at the 5/30% interface, as described before (54). 26% or 35% of cell-associated StxB was found in DRMs in mesangial cells or GMVECs, respectively (figure 4), similar to the 30% that were DRM associated in HeLa cells (not shown). After preincubation with TNF-α for 24 hr, no significant change in percentages of StxB in the DRM fraction was observed.

Figure 4 Analysis of StxB association with DRMs



Vertical: Stx/StxB (% of total)
Horizontal: Fractions

After 24 hours preincubation with (blue) or without (white) TNF-α, iodinated StxB-Glyc-KDEL was bound to human GMVECs (A) or human mesangial cells (B) on ice. The cells were washed, lysed in 1% Triton X-100 buffer, and DRMs were floated in Optiprep step gradients. The gradients were fractionated and fractions were counted in a gamma counter. StxB was readily detected in the DRM fraction 2 in both cell types.

Discussion

During the development of HUS, most extensive tissue damage occurs within the kidney (143). Proximal tubular cells are exquisitely sensitive to Stx, undergoing apoptosis even without prestimulation with TNF-α or lipopolysaccharide (LPS) (144). Nevertheless, it is generally accepted that Stx mediated endothelial cell damage is the major event triggering the development of HUS (143, 145). It has been shown that cytokines can up-regulate the expression of Gb3 in both mesangial cells and GMVECs (132, 133, 134). These findings have been confirmed in our experiments with TNF-α. Stx does not affect mesangial cell viability under basal conditions or after preincubation with TNF-α, whereas protein synthesis is inhibited (133, 134). In unstimulated GMVECs, Stx does not inhibit cell viability and protein synthesis, but GMVECs become highly sensitive for Stx after stimulation with TNF-α (132).

In this study, we have demonstrated that the receptor binding B-subunit of Shiga toxin/Stx-1 follows the retrograde route in TNF-α stimulated and unstimulated mesangial cells and GMVECs. The B-subunits are organized in a pentameric form (146). The presence of the Shiga toxin A-subunit of cell surface-bound toxin stimulates clathrin-dependent uptake of the toxin (111). However, the trafficking route of the B-subunit and the holotoxin is the same (62). Although the expression of Gb3 is up-regulated after incubation with TNF-α, and TNF-α-treated cells bind more Shiga toxin/Stx-1 than unstimulated cells, no change in the retrograde transport efficiency of Shiga toxin/Stx-1 is observed. TNF-α therefore seems to increase cytotoxicity of Shiga toxin/Stx-1 without affecting the efficiency of the toxin's intracellular trafficking. The relative transport rate of Shiga toxin/Stx-1 remains unchanged. Interestingly, it has also been shown that Stx-1 does not influence the viability of mesangial cells and unstimulated GMVECs (132, 133), despite its retrograde transport in these cells, as shown in this study. We conclude that whether or not a cell is sensitive to Shiga toxin/Stx-1 does not only depend on retrograde transport (see below), but also on additional factors. One of these might be the amount of Shiga toxin/Stx-1 that reaches the cytosol. Gb3 expression of unstimulated GMVECs is much lower than the expression of Gb3 on mesangial cells. When both are stimulated with TNF-α, Gb3 expression of GMVECs increases more and reaches higher levels as in mesangial cells (table 1). Under these conditions, a threshold level of Shiga toxin/Stx-1 may be reached in the ER that permits a cytotoxic amount of Stx to be transported to the cytosol. Cellular processing of the uncleaved toxin by the enzyme furin has also a role in this toxicity (132). It cannot be excluded, however, that TNF-α has additional cellular effects that also contribute to efficient intoxication of cells, such as for example increasing retrotranslocation of toxin to the cytosol.

When a toxic dose of Shiga toxin/Stx-1 reaches the cytosol of a cell, ribosomes are inactivated and protein synthesis is massively inhibited which leads to cell death (132). Classically, it was thought that this was the only relevant biologic activity of Shiga toxin/Stx-1 (17, 132, 133). It is only recently that research in this domain has focused on what is called the 'ribotoxic stress model'. When Shiga toxin/Stx-1 reaches the cytosol in a subtoxic dose, e.g. as in mesangial cells or unstimulated GMVECs, only part of the ribosomes are inactivated. The intact ribosomes are able to keep up with protein synthesis so that cell viability is not affected. This Shiga toxin/Stx-1-mediated damage to ribosomes may generate a cellular-stress signaling response leading to production of new ribosomes and proinflammatory cytokines (56, 117, 147). In human umbilical vein endothelial cells (HUVECs), which are comparable to GMVECs in that they are only sensitive to Shiga toxin/Stx-1 after preincubation with TNF-α (148), stimulation with a subtoxic dose of Stx-1 leads to expression of genes that encode for chemokines and cytokines (149). This response is also described in a monocytic cell line (56) and intestinal epithelial cells (31, 117). The ribotoxic stress model might be applicable to renal cells, and thus contribute to the pathogenesis of HUS.

In conclusion, we describe that in human GMVECs and mesangial cells, Shiga toxin/Stx-1 is transported via the retrograde route to the Golgi apparatus and ER. This study thereby closes one of the remaining gaps in the understanding of the pathological process of HUS: it describes the transport route of Shiga toxin/Stx-1 in its physiological target cells, i.e. human glomerular endothelial and mesangial cells.

Our study shows that TNF-α induced sensitization of renal cells must result from higher amounts of cell-associated toxin rather than from stimulation of retrograde transport as such, and thus, makes an important contribution to the understanding of the cellular mechanisms of HUS.

In conclusion, we describe that in human GMVECs and mesangial cells Shiga toxin/Stx-1 is transported via the retrograde route to the Golgi apparatus and ER.

Since WPbs contain regulators for both haemostasis and inflammation, we evaluated whether stimulation of primary human endothelial cells (HUVECs and GMVECs) with a subtoxic dose of Stx could trigger exocytosis of WPbs.

Chapter 3.2
The effect of Shiga toxin on Weibel-Palade bodies in primary human endothelial cells

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Submitted

Abstract

Diarrhea-associated haemolytic-uraemic syndrome (D+HUS) is associated with the presence of exotoxin Shiga toxin (Stx). In plasma of patients with D+HUS, levels of von Willebrand factor (VWF) are elevated as a result of renal endothelial damage. In endothelial cells, VWF is stored in secretory granules called Weibel-Palade bodies (WPbs). The aim of this study is to examine the possible triggering effect of Stx1 on the endothelial cells, with particular reference to the exocytosis of WPbs.
Cultured human primary endothelial cells (HUVECs and GMVECs) were stimulated by thrombin and Stx1 in static conditions as well as in flow. The amount of secreted VWF in the supernatant as well as the remaining intracellular fraction was determined.
In static conditions, Stx could not induce a significant exocytosis of WPbs in HUVECs as shown by both methods. On the contrary, stimulation of HUVECs by Stx in flow resulted in a significant decrease in the amount of intracellular VWF. No effect of Stx on HUVECs in flow was observed. In 2 out of 4 investigated GMVECs the amount of intracellular VWF was decreased by Stx during flow. Stx might contribute via an effect on WPbs to an increased exocytosis of VWF in flow.

Introduction

The exotoxin Shiga toxin (Stx), originating from specific serotypes of *Escherichia coli*, is the central virulence factor in diarrhoea-associated haemolytic-uraemic syndrome (D+HUS). D+HUS is the most important cause of acute renal failure in children. In the new classification of HUS, infections due to Stx- producing bacteria, belong to the category of 'etiology advanced' (2). The underlying pathogenic mechanism is the endothelial damage of the glomeruli and arterioles in the kidney by Stx (1). This damage leads to the formation of thrombi and initiation of an inflammatory response. Since coagulation plays an important role in this disease, much research has been performed to elucidate possible disturbances in this system. Von Willebrand Factor (VWF) antigen was found in an increased level in serum of patients in the acute phase of D+HUS. A loss of large multimers and in increase of small multimers was observed. This may be due to abnormal shear stress in the microvascular circulation (150).
VWF is involved in direct adhesion and aggregation of platelets on damaged endothelium, whereas it also functions as a chaperone for coagulation factor VIII. Wagner *et al* discovered that VWF was stored in endothelium-specific storage vesicles, called Weibel-Palade bodies (WPbs) (151). The WPbs are rod-shaped cytoplasmic structures with a tubular composition. After perturbation of the endothelial cell, WPbs can be exocytosed through two different mechanisms (152). One pathway is activated by an increase on intracellular calcium and will lead to both exocytosis of the WPbs and the formation of stress fibers with loss of endothelial cell barrier function. For an example, thrombin uses this mechanism. The other pathway is regulated by cAMP and will also lead to exocytosis, but in contrast, improves the endothelial cell barrier function. Possible agonists of this route are vasopressin and epinephrine. The work of Ikeda *et al* described that Stx increased calcium in Vero cells, which are highly sensitive to its effect (153).
Many different components of the WPbs have been identified, and these indicate the key role that WPbs play in many important physiological processes in endothelial cells. Its constituents are involved in haemostasis (VWF and tissue-type plasminogen activator), induce vasoconstriction (endothelin-1, endothelin converting enzyme), and inflammation (P-selectin, eotaxin-3, IL-8, angiopoietin-2, CD63, α1,3-fucosyltransferase VI, osteoprotegerin) (152). With all these different components the endothelial cell is capable of a rapid response to changing conditions. This is in contrast to the slower response with *de novo* synthesis.
Many inducers of exocytosis of WPbs have been described, but in this work we investigated if Stx could also trigger its release. Since WPbs contain regulators for both haemostasis and inflammation, we evaluated whether stimulation of primary human endothelial cells (HUVECs and GMVECs) with a subtoxic dose of Stx could trigger exocytosis of WPbs. For this purpose, we have determined the effect of Stx on the quick release of the haemostatic VWF. The effect on another component of the WPbs, angiopoietin-2 (ANG) was included.

Methods

Culture of endothelial cells

Human umbilical venous endothelial cells (HUVECs) were harvested after collagenase (Worthington: NJ, USA) treatment as previously described (121). HUVECs in passage 2-4 were used. Glomerular microvascular endothelial cells (GMVECs) were obtained from human kidneys (collected after informed consent). Glomeruli were isolated under sterile conditions using a gradual sieving procedure followed by digestion with collagenase (132). GMVECs in passage 7-10 were used. Cells were grown confluent on tissue culture plastic plates coated with 1% porcine gelatine (Fluka: Neu-Ulm, Germany). Medium (M199; Gibco: Paisley, UK) with addition of 10% human serum (Cambrex: Walkersville, USA), 10% newborn calf serum (Gibco: Paisley, UK), 1% 200 mM L-glutamine (MP Biomedicals; Eschwege/Germany), 1% penicillin-streptomycin (Gibco: Paisley, UK), growth factor (extracted from bovine brains (119)) and 0.1% heparin (Leo Pharma BV: Breda The Netherlands) was refreshed every two days.

Quantification of VWF in supernatant endothelial cells

To study the exocytosis of WPbs in the supernatant of endothelial cells, the cells were grown to confluency in 24 wells plates (Corning Incorporated: Schiphol-Rijk, The Netherlands). During 5, 10 and 15 minutes, the cells were incubated with thrombin (5 U/ml; Organon Technika, Turnhout, Belgium) or Stx1 (10 nM; Stx1 was kindly provided by Dr. M. Karmali, Toronto, Canada) which was endotoxin-free as determined with a Limulus-assay, at 37˚C. Cells remained viable during this incubation, as determined with a 3H-leucine incorporation assay (data not shown). The stimuli were dissolved in M199 with 2.5% fetal calf serum (Greiner Bio-One; Kremsmunster, Austria). Cells were rinsed once with M199 before adding the stimuli. After the incubation the supernatant was removed, centrifuged at 2000 xg and stored immediately at –20 ˚C.

The amount of released VWF in the supernatant from endothelial cells was measured by ELISA. All experiments were performed in duplicate.

The ELISA for VWF was performed according to Ingerslev *et al* (154). 96 wells-plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-human VWF polyclonal antiserum (Dako A/S: Glostrup, Denmark). Test samples were diluted 1:100 and added to the ELISA plate. Pooled plasma (10 µg/ml) was used as standard. After incubating for 2 hours at room temperature, the plate was washed and rabbit anti-human VWF peroxidase conjugate (Dako A/S, Glostrup, Denmark) was added. Again, after 2 hours the plate was properly washed and substrate solution (ortho-phenylenediaminetablets (Dako A/S, Glostrup, Denmark) and H_2O_2) was added to each well. The reaction was stopped after 30 minutes with H_2SO_4 and the extinctions were measured at a wavelength of 492 nm in a BioRAD plate reader.

Quantification of intracellular VWF and ANG in endothelial cells

Endothelial cells were cultured as described in the above section. Stimulation was performed in both flowing and static conditions. In both set-ups, cells were exposed to thrombin (1 U/ml), Stx1 (10 nM) or PMA (phorbol 12-myristate 13-acetate (Fluka: Neu-Ulm, Germany); 100 ng/ml) during 15 minutes (flowing conditions) or 1 hour (static conditions) in EBM-2 with single quots (Lonza, Brussels, Belgium) and M199 with 0.5% BSA. Before the experiment, cells were kept overnight with EBM-2 + single quots and 5% FCS. To apply shear stress, cells were incubated with the stimulants in a parallel Focht flow perfusion chamber with well-described characteristics (155). A coverslip with a monolayer of endothelial cells was assembled in the flow chamber, which was mounted on an inverted phase-contrast microscope stage (Axiovert 35M, Zeiss, Germany). The stimulus was prewarmed to 37˚C and aspirated from a reservoir through the perfusion chamber with a withdrawal syringe pump (Harvard apparatus, Holliston, MA, USA; ANTEC, Leiden, The Netherlands). The complete system was present in a 37˚C incubator. Temperature fine-tuning was performed by the Bioptechs FCS2 controller. The applied shear stress was 1 dyne/cm^2 similar to Nolasco *et al* (156). After the incubation with the stimulants, the cells were lysed according to Michaux *et al* (157). After rinsing the cells once with PBS, lysis buffer (M199 with 0.2% BSA and 0.1% Triton X-100 (Sigma-Aldrich, Zwijndrecht, The Netherlands)) containing protease inhibitors (aprotinine 30 µl/ml, PMSF, 20 µl/ml, ortho-vanadate 100 mM 10 µl/ml) was added at 4˚C during 30 minutes. Afterwards, lysates was centrifuged during 5 minutes at 10000xg at -4˚C. For imaging, cells were fixed in the flow chamber immediately after the experiment (flow conditions) or in a 24-well plate (static conditions).

The lysates were then analysed for the presence of VWF and ANG. VWF was detected with an ELISA as described above for the supernatants. Also, ANG was determined with an ELISA-procedure. A 96-wells plate was coated over night with a monoclonal antibody against ANG (R&D systems, Oxon, United Kingdom) at room temperature. After washing with PBS/Tween, the wells were blocked with PBS containing 1% BSA, 5% sucrose (Fluka: Neu-Ulm, Germany) and 0.05% sodiumacetate (Fluka: Neu-Ulm, Germany) for 1 hour at room temperature. After blocking, the samples were added to the wells during 2 hours at room tempera-ture. Biotinylated anti-human ANG (R&D systems, Oxon, United Kingdom) in 1% heat inactivated mouse serum was used during 2 hours at room temperature. After rinsing, streptavidin HRP (R&D systems, Oxon, United Kingdom) was applied for 20 minutes at room temperature followed by OPD. Subsequently, extinctions were determined with a plate reader.

Furthermore, cells were prepared and analysed by confocal laser scanning microscopy as described by Romani-de Wit *et al* (158). Cells were stained for VWF using monoclonal antibody CLB-RAg20 (159) and for CD31 using monoclonal antibody CLB-HEC-75 (160). Alexa 594 and-633 conjugated secondary antibodies were from Invitrogen (Breda, the Netherlands). Laser Scanning Confocal Microscopy was performed using a Zeiss LSM510 and results were analyzed using Zeiss LSM510 version 4.0 software.

Statistics

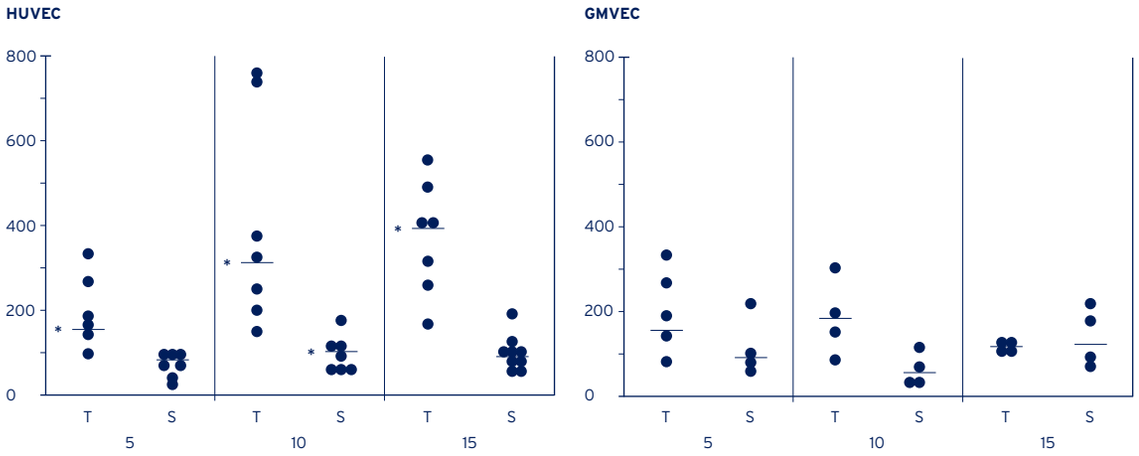
All data presented are expressed as a range with the median. Significance of the quantitative data was analyzed using the Wilcoxon signed ranks test. The statistical level of significance was defined as $P< 0.05$.

Results

Quantification of VWF in the supernatant of endothelial cells

To study the possible exocytosis of WPbs from human endothelial cells after incubation with a subtoxic dose of Stx, the presence of VWF in the supernatant of stimulated endothelial cells (HUVECs and GMVECs) was determined. Thrombin was used as a positive control.

Figure 1 VWF in supernatant of HUVECs and GMVECs after incubation with thrombin or Stx1



Vertical: VWF of control (%)
Horizontal: Incubation time (min)

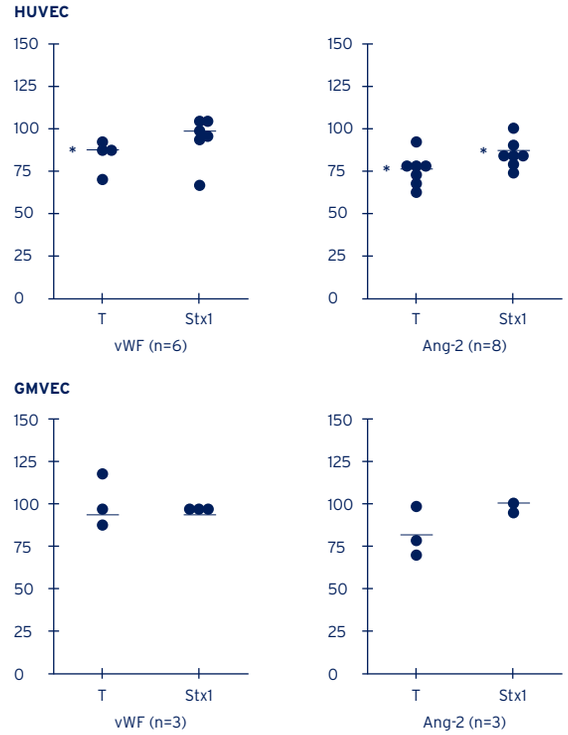
HUVECs (n=7) and GMVECs (n=4) were incubated with thrombin or Stx1 for respectively 5, 10 and 15 minutes. Negative control was set at 100%. Thrombin clearly triggers secretion of VWF in HUVECs, whereas Stx1 does not induce a release. In GMVECs both thrombin and Stx do not induce a significant effect. The bar depicts the median. The asterisk (*) indicates a significant effect (P<0.05).

Figure 1 presents the data on the level of VWF in the supernatant determined with ELISA. The results are expressed as percentage of the negative control. Thrombin was able to induce a significant release of VWF after 5, 10 and 15 minutes in HUVECs. Only after ten minutes of incubation of HUVECs with Stx, there is a small but significant increase of VWF (median control value 100% versus 125% with Stx). In the small number of investigated GMVECs, neither thrombin nor Stx1 caused a significant release of VWF.

Quantification of intracellular VWF and ANG in endothelial cells

To determine the level of intracellular VWF after incubation with Stx1, cell lysates were analysed by ELISA. If Stx1 is able to induce exocytosis, the amount of VWF should be decreased compared to a negative control. Also, the presence of ANG was determined, since this is also a constituent of WPbs.
Figure 2 represents the data of the experiments performed in static conditions. HUVECs stimulated by the positive control, thrombin, contain a clear decreased level of VWF and ANG. In contrast, Stx-1 was not able to exocytose VWF although a small decrease of ANG in HUVECs is observed. GMVECs are not sensitive for the effect of Stx in static conditions. No decrease in VWF or ANG could be detected in this small study. To illustrate this, figure 3 demonstrates confocal images of GMVEC after incubation with thrombin and Stx1. Thrombin decreases the amount of WPbs, whereas Stx1 does not have an effect.
Experiments were also performed in flowing conditions since it approaches more the *in vivo* situation. Figure 4 represents our data of HUVECs incubated with thrombin or Stx1 in flow (n=5). In this set-up, Stx1 is triggering the endothelial cells into a significant decrease of VWF. ANG is also decreased, but non-significantly. Table 1 demonstrates our data gathered after incubation of GMVEC in flow. 4 GMVECs donors were used. Donors 1 and 3 respond with a decrease of VWF and ANG after incubation with both PMA and Stx1. Only in these experiments PMA was used as a stimulus. Thrombin had a similar but less pronounced effect. However, donors 2 and 4 did not respond adequately to the positive control. This suggests that if the donor is sensitive for the positive control, it will also respond by the release of VWF and ANG. But not every donor of GMVECs is sensitive for the positive control. This is also demonstrated in figure 5 that demonstrates 2 different GMVECs-donors of which donor A responds to the positive control and donor B does not.

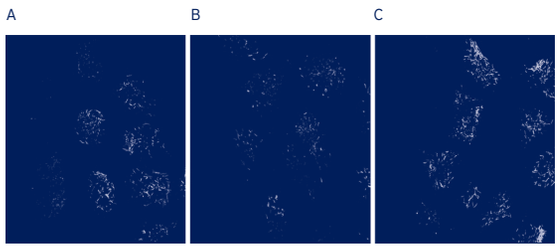
Figure 2 Intracellular VWF and ANG in HUVECs and GMVECs



Vertical: % of control

HUVECs and GMVECs were incubated with thrombin (T) and Stx1 in static conditions. After the incubations, the intracellular amounts of VWF and ANG were calculated.
Thrombin is capable of decreasing the intracellular amount of VWF and ANG in HUVECS, whereas it does not have a significant effect in GMVECS. Stx1 significantly decreases ANG in HUVECs. No effect of Stx1 is detected in GMVECs. The asterisk (*) indicates a significant effect (P<0.05).

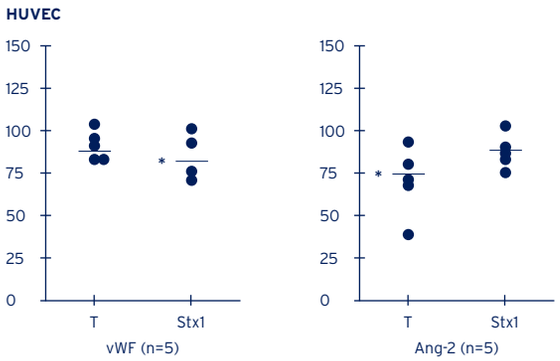
Figure 3* Confocal microscopy of GMVECs for VWF after incubation with thrombin or Stx1 in static conditions



*** To see figure in full colour, see Appendix**

GMVECs was incubated under static conditions with thrombin and Stx1 (A= control; B= thrombin; C= Stx). Cells were fixed and VWF was stained using monoclonal antibody CLB-Rag20. Z-stack images were made and projections are shown. Confocal imaging showed the same amount of WPbs after incubation with Stx1 compared to the control. Thrombin induced a clear effect.

Figure 4 Intracellular VWF and ANG in HUVECs in flow



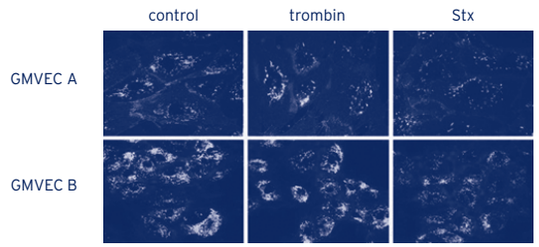
Vertical: % of control

HUVECs were incubated with thrombin (T) and Stx1 in flowing conditions. After the incubations, the intracellular amounts of VWF and ANG were calculated. Stx1 is capable of decreasing the intracellular amount of VWF, whereas thrombin has a smaller and non-significant effect. However, thrombin significantly decreases ANG. Stx1 can not produce a significant effect on ANG. The asterisk (*) indicates a significant effect (P<0.05).

Table I VWF and ANG in GMVECs incubated in flow

	VWF (% of control)						ANG (% of control)					
Stimulus	**Donor**				**statistics**		**Donor**				**statistics**	
	1	**2**	**3**	**4**	**median**	**p-value**	**1**	**2**	**3**	**4**	**median**	**p-value**
PMA	75	99.1	65	99.3	87.1	0.068	76.4	84	83.8	95.8	83.9	0.068
Stx1	82	104	88.9	93.3	91.1	0.144	82.6	112.3	93.1	102.2	97.7	0.715

Figure 5* Confocal microscopy of GMVECs incubation with thrombin or Stx1 in flow conditions



*** To see figure in full colour, see Appendix**

GMVEC donor A and B were incubated in flow with thrombin and Stx1. Cells were fixed and stained for VWF (blue) and CD31 (light blue). The merge of fluorescent signals is shown. GMVECs B show less effect of thrombin than GMVECs A. A noticeable effect can be detected in both donors after exposure to Stx.

Discussion

In this paper, the effect of Stx1 on the exocytosis of the WPbs was studied. The components of the WPbs play a central role in coagulation and inflammation of endothelial cells. Stx1 is a key virulence factor in D+HUS. The pathogenesis of this disease is based on the damage of the glomerular endothelium with well-described consequences on the coagulation and inflammation. If Stx1 proved to be an inducer of exocytosis of WPbs, this can contribute to our insight in the pathogenesis of D+HUS.

As a marker for exocytosis of WPbs, we have measured the presence of VWF in the supernatant of HUVECs and GMVECs. The remaining intracellular fractions of WPBs-constituents VWF and ANG were also determined after the incubations. In HUVECs, there is a clear effect of thrombin and also Stx1 induces a small release of VWF after 10 minutes in the supernatant. Since this effect is quick, it can be attributed to the release of WPbs. The intracellular content of VWF was not decreased by Stx1. This confirms the work of Kaye *et al* (161) which describes the positive effect of TNF-α on the release of WPbs, whereas a four-hour incubation of HUVECs with Stx did not induce any release. WPbs were detected with immunofluorescence microscopy using antibodies against VWF.

We would like to stress that in our experimental settings, especially in working with primary GMVECs and HUVECS, we have experienced donor variability for the sensitivity of Stx and thrombin.

To approach the *in vivo* situation, we have performed equal experiments with exposure of the endothelial cells to shear stress. Especially, since Nolasco *et al* described the secretion of ultra-large multimeric strings of VWF after stimulation of endothelial cells with Stx in flow conditions similar to our experiments (156). Also, Galbusera *et al* published an article in which they describe the stimulating effect of prolonged exposure to high shear stress on the release of VWF (162). HUVECs and GMVECs were incubated with a positive control (thrombin or PMA) and Stx1 in flow. After these incubations, the intra-cellular amount of VWF and ANG was measured. With these conditions of shear stress, HUVECs could be triggered into a significant decrease of VWF by Stx1. Interestingly, the GMVECs donors that responded to the positive control, were also able to respond to Stx1 as can be seen in table 1. This donor variability is also demonstrated in figure 5.

Some important conclusions can be drawn from these experiments; 1) Thrombin can induce the release of WPbs in HUVECs; 2) In HUVECs, Stx1 appears to have a significant effect on WPbs in flow condition, and not in static conditions; 3) There is a possible influence of flow on the effect of Stx on GMVEC.

Further studies under flow conditions will reveal new insight in the pathogenesis of HUS, which can not be obtained under static conditions.

Further studies under flow conditions will reveal new insights in the pathogenesis of HUS, wich can not be obtained under static conditions.

The performed experiments allow us to evaluate the effect of Stx on firm leukocyte-adhesion under dynamic flow conditions and whether HSPG are involved.

Chapter 3.3
Shiga toxin induced firm adhesion of human leukocytes to endothelium is in part mediated by heparan sulfate

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Abstract

Background

Shiga toxin (Stx) is the main pathogenic factor in the haemolytic-uraemic syndrome (HUS). Stx damages the renal endothelium which leads to inflammation and coagulation. Endothelial heparan sulfate proteoglycans (HSPG) and in particular heparan sulfate, play an important role in the inflammatory process by acting as a ligand for L-selectin. Furthermore, leukocytes are able to interact with chemokines bound to HSPG (examples are IL-8, RANTES and MCP-1). This leads to an activation of integrins on leukocytes and results in more stable leukocyte-endothelial wall adhesion.
In this study, we have evaluated the effect of a subtoxic dose of Stx1 and -2 on the HSPG and its role in adhesion of leukocytes.

Methods

Primary human umbilical venous endothelial cells (HUVECs) and primary human glomerular microvascular endothelial cells (GMVECs) were incubated for 24 hours with a subtoxic dose of Stx1 or -2. Then, cells were treated with heparan sulfate-degrading enzyme heparitinase I or left untreated, followed by determination of binding leukocytes to endothelial cells in a parallel plate flow chamber.

Results

In both cell types, Stx increased the amount of firmly adherent leukocytes. After removal of endothelial heparan sulfate, the number of adhering leukocytes decreased.

Conclusions

HSPG have a distinctive role in adhesion of leukocytes to endothelial cells stimulated by a subtoxic dose of Stx.

Introduction

Haemolytic-uraemic syndrome (HUS) is a form of acute renal failure in children, combined with thrombocytopenia and haemolytic anaemia. Although new insight has been gained in the pathogenesis of genetic variants of HUS, the underlying mechanism in infection-related HUS is still not understood (163). The most important pathogen in infection–related HUS is *Escherichia coli* O157:H7, which produces Shiga toxin (Stx) (1). There are two different variants, Stx1 and -2, which share 50% homology and have a similar biological effect. This toxin has a cytotoxic effect on the renal endothelium. It internalises after binding to a specific receptor (CD77; Gb3), and disables the ribosome (163). This undermines the protein synthesis of the cells and will ultimately lead to cell death. Because of this damage, two different biological cascades are initiated: coagulation and inflammation. Renal biopsies of patients with HUS show occluded glomerular arterioles and deposition of inflammatory cells (163).
During the last decade it has become evident that heparan sulfate proteoglycans (HSPG) are crucial for leukocyte-endothelium trafficking (164, 165). These negatively charged structures are ubiquitously expressed on cell surfaces, blood cells and in the extracellular matrix (164, 165). HSPG is a macromolecular structure consisting of a protein backbone covalently carrying heparan sulfate (HS) chains. HS belongs to the family of glycosaminoglycans that comprises the highly sulfated heparin and non-sulfated hyaluronan. The HS chain shows a high diversity in structure, which is dictated by combinations of possible modifications such as sulfation patterns. This allows HSPG to interact with many different proteins like cytokines, chemokines and cell-adhesion molecules. HSPG on endothelial cells play an important role in tethering, rolling, firm binding and transmigration of leukocytes to the extracellular matrix (164, 165). After an inflammatory insult, endothelial cells will express more cell adhesion molecules (E- and P-selectin), which will bind to leukocytes that express ligands such as P-selectin glycoprotein ligand-1. During the rolling phase, endothelial HS will serve as a ligand for L-selectin on leukocytes. They will roll over the endothelial cells, and get into contact with endothelial HS-bound chemokines, which in their turn activates integrins on leukocytes like MAC-1. Subsequently, leukocytes will adhere more firmly, which is in part mediated by the HS-MAC-1 interaction. Finally, the leukocytes will transmigrate through the endothelium to the extracellular matrix.
Recently, Wang *et al* described a reduced neutrophil infiltration in an experimental peritonitis in genetically engineered mice with low sulfation of endothelial HSPG (166). Furthermore, Rops *et al* have demonstrated that HS on mouse glomerular endothelium activated by TNFα, is crucial for the number of rolling and firmly adhering leukocytes (167).
In this study, we focused on the inflammatory process induced by Stx in HUS. The performed experiments allow us to evaluate the effect of Stx on firm leukocyte adhesion under dynamic flow conditions and whether HSPG are involved. Since application of shear stress changes the phenotypic appearance of endothelial cells, these experiments approach the *in vivo* situation (168). Endothelial cells from umbilical origin and glomerular endothelial cells were used. The latter were selected as being the primary target of shiga toxin.

Material & Methods

Culture of endothelial cells

Human umbilical venous endothelial cells (HUVECs) were harvested after collagenase treatment (Worthington: NJ, USA) and cultured according to a previous described method (121). HUVECs from 5 donors (passage 2-4) were used. Glomerular microvascular endothelial cells (GMVECs) were obtained from human kidneys and cultured as described previously (132). GMVECs from 3 donors in passage 7-10 were used. Every donor was used once in the experiments. For the adhesion experiments, cells were seeded on plastic coverslips (Thermanox; Nunc Inc., Naperville, USA). Growth medium of both cell types consisted of M199 (Gibco: Paisley, UK) with addition of 10% human serum (Cambrex: Walkersville, USA), 10% newborn calf serum (Gibco: Paisley, UK), 1% 200 mM L-glutamine (MP Biomedicals; Eschwege/Germany), 1% penicillin-streptomycin (Gibco: Paisley, UK), growth factor (extracted from bovine brains (119)) and 0.1% heparin (Leo Pharma BV: Breda, The Netherlands). Medium was refreshed every two days.

Isolation of leukocytes

Leukocytes were isolated from fresh human venous blood collected from healthy volunteers after informed consent as described previously (169). Briefly, after dilution of the blood with cold saline, it was centrifugated at 200 xg for 10 minutes at 4˚C. Subsequently, the cell pellet was gently put on 4 volumes of Emagel (Boehringwerke AG; Marburg, Germany) in order to sediment the erythrocytes. This was performed for 45 minutes at 4˚C. The supernatant was removed and centrifugated at 4˚C for 10 minutes at 500 xg and the pellet was washed twice by centrifugation with saline. Then ammonium chloride was added for lysis of the remaining erythrocytes. After new washing steps with saline, cells were resuspended in culture medium at a concentration of 1 x 10^6 cells per ml. For every experiment the leukocytes of 1 donor were used.

Incubation and manipulation of endothelial cells

Endothelial cells were incubated for 24 hours with a subtoxic dose (50 pM) of Stx1 (kindly provided by Dr. M. Bielaszewska, Munster, Germany). This dose does not exert a cytotoxic effect, which was confirmed with trypan blue exclusion. 50 pM of Stx1 was free of lipopolysaccharide (LPS) as assessed with a Limulus-assay (detection limit 0.006 ng/ml). Stx2 (Toxin Technology Inc, Sarasota, USA) was also used in a subtoxic dose (50 pM), since this toxin was able to increase the adhesion of leukocytes as demonstrated by the work of Zoja *et al* (170). As the experiments were performed in the same laboratory, the toxin was produced equally as described by Zoja *et al* (12). It contains 117 pg LPS/µg Stx2, which is 0.41 pg/ml LPS at a dose of 50 pM Stx2. This is below the detection limit of the Limulus assay. This indicates that LPS does not influence the final effects observed in the cells after stimulation with Stx1 and Stx2.

After incubation with Stx, HS was cleaved off by the HS-degrading enzyme heparitinase I (Seikagaku Co; Tokyo, Japan). This enzyme cleaves the alpha-N-acetyl-D-glucosaminidic linkage in heparan sulfate, producing disaccharides. Briefly, the enzyme was dissolved in 0.5 U/ml in binding buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 4 mmol/l KCl, 11 mmol/l D-glucose and 0,1% bovine serum albumin (BSA), pH 7,4), and incubated for 30 minutes at 37˚C.

The efficacy of the enzyme was evaluated with immunofluorescence and FACS-analysis with antibodies directed against heparan sulfate (primary antibody: mouse anti-human heparan sulfate 10E4 (US Biological, Swampscott, USA); secondary antibody: goat anti-mouse FITC conjugated (Jackson Immunoresearch, West Grove USA). For FACS-analysis, cells were detached by using EDTA 10 mM.

Adhesion assay under dynamic flow conditions

A parallel-plate flow chamber was used to apply shear stress. This system is described in the article of Morigi *et al* (169). In short, cover slips with a monolayer of endothelial cells were placed in the flow chamber. First, cell-free medium with 0.5% BSA was flowed over the cells during 2 minutes at 0.6 dynes/cm^2 for equilibration. Then, the leukocyte suspension was perfused through the chamber with 1.5 dynes/cm^2 during 10 minutes to approach the level of shear stress in glomerular arterioles. This was followed by again cell-free medium at 3 dynes/cm^2 during 5 minutes. At this higher level of shear stress, the number of firmly adhering leukocytes can be analyzed. The experiment was visualized and recorded with a video recording system connected to an inverted phase-contrast microscope. The number of firmly adhering cells was calculated in 10 random equal fields by 2 observers.

Statistical analysis

Results are expressed as mean ± SD. Data was analyzed with student's T-test. Statistical significance level was defined as $P<0.05$.

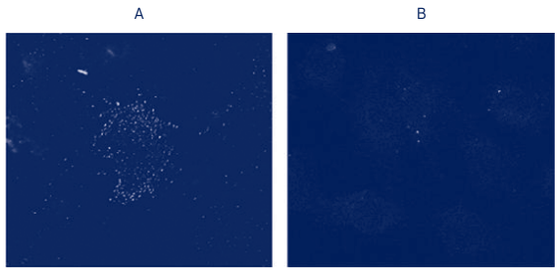
Results

Cleavage of HS from endothelial cells

To evaluate the efficacy of the HS-cleaving enzyme, the amount of HS after treatment with heparitinase I was estimated with immunofluorescence and FACS-analysis.

The immunofluorescence staining of endothelial cells by the anti-HS antibody 10E4 is shown in figure 1A. Removal of HS by heparitinase I revealed a complete abolishment of staining (figure 1B). To quantify these results, we repeated this analysis by FACS. This confirmed the immunofluorescence staining, since treatment of the cells with the enzyme strongly reduced the staining (figure 2).

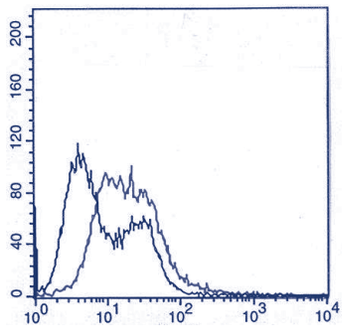
Figure 1* Efficiency of heparitinase I treatment determined by immunofluorescence staining



*** To see figure in full colour, see Appendix**

Removal of HS on HUVECs was determined by immunofluorescence staining with the anti-HS antibody 10E4. HS on HUVECs was stained without (A) and after (B) treatment with heparitinase I.

Figure 2 Efficiency of heparitinase I treatment determined with FACS analysis



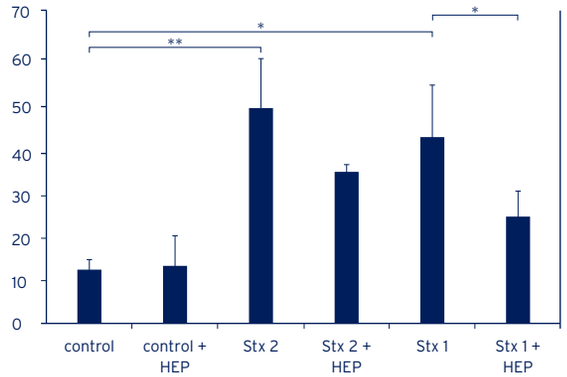
Vertical: counts
Horizontal: FL1-H

The amount of HS on HUVECs was analyzed after treatment with heparitinase I. The plot in light blue represents the cells without treatment of heparitinase I. The dark blue plot represents the cells after treatment with heparitinase I.

Flow experiments

After incubation of both endothelial cells with Stx1 and -2, there clearly was an increased number of firmly adhering leukocytes to HUVECs (range: Stx1 13-55; Stx2 38-70) (figure 3) or GMVECs (range: Stx1 52-60; Stx2 51-62) (figure 4). Removal of HS from endothelial cells that were not incubated with Stx, did not have a significant effect on firm leukocyte adhesion. However, treatment of the Stx-incubated endothelial cells with heparitinase I clearly revealed a decreased number of firmly adhering leukocytes (range: HUVEC Stx1 18-35; HUVEC Stx2 28-63; GMVEC Stx1 34-37; GMVEC 32-39) (figure 3 and 4). Taken together, removal of HS from Stx-incubated endothelial cells decreased the number of firmly adhering leukocytes.

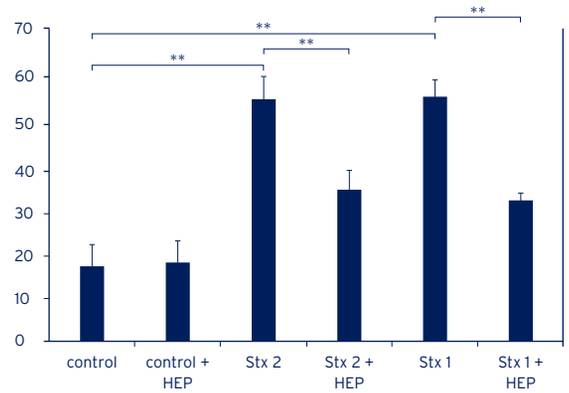
Figure 3 Number of firm adherent leukocytes on HUVECs



Horizontal: Incubation
Vertical: cells/mm^2

HUVECs (n=5) was incubated for 24 hours with Stx1 or -2. Furthermore, they were treated with heparitinase I (HEP) or remained untreated. The number of firmly adherent leukocytes was calculated. Stx increased the amount of firmly adhering leukocytes. Removal of HS decreased this effect.
* <P0.05; **<P0.01

Figure 4 Number of firm adherent leukocytes on GMVECs



Horizontal: Incubation
Vertical: cells/mm^2

GMVECs (n=3) was incubated for 24 hours with Stx1 or -2. Furthermore, they were treated with heparitinase I (HEP) or remained untreated. The number of firmly adhering leukocytes was calculated. Stx increased the amount of adherent leukocytes. Removal of HS can decreased this effect.
* <P0.05; **<P0.01

Discussion

We showed that HS play an important role in the adhesion of leukocytes to endothelial cells after treatment with a subtoxic dose of Stx. After removal of endothelial HS from Stx-incubated endothelial cells, the number of firmly adherent leukocytes is clearly decreased.

Since Stx is considered a cytotoxic toxin, our results are very interesting. The increase in adhesion of leukocytes indicates that there must also be a stimulatory effect, besides inhibition of the protein synthesis. Several mechanisms for our results can be suggested. One possibility is an increased expression of HSPG after incubation with a subtoxic dose of Stx. Another option is an increase in bound chemokines on endothelium-HSPG after treatment with Stx. This will result in more adhering leukocytes. The basal level of HSPG present on endothelial cells will be sufficient to bind additional released chemokines. Zoja *et al* described an NF-κB dependent up-regulation of mRNA from IL-8 and MCP-1 by Stx and inhibition of leukocyte adherence after the application of antibodies against IL-8 and MCP-1 (170). The work of Matussek *et al* confirmed the up-regulation of mRNA of pro-inflammatory proteins by Stx (149). When released chemokines bind to HSPG on endothelial cells, they are protected from proteolysis, and are able to rapidly induce activation of integrins on leukocytes (164).

By using the parallel-flow chamber, we have applied shear stress to the endothelial cells, which mimics the *in vivo* situation. It is possible that the combination of Stx exposure and shear stress will increase the biological effect of Stx on endothelial cells. Moreover, during the development of HUS in patients, leukocytes like neutrophils and monocytes will become activated (112, 171). This will lead to increased serum-levels of different cytokines. In our experiments, leukocytes from healthy donors in serum-free medium were used. Possibly, *in vivo* there will be a much stronger effect of Stx in combination with cytokines from activated leukocytes.

The residual number of firmly adhering leukocytes after removal of HS from Stx-incubated endothelial cells, indicate that also HS-independent factors are involved in the increased adhesion of leukocytes. Fractalkine could be such a factor, since Ramos *et al* demonstrated a selective depletion of mononuclear leukocytes expressing the receptor for fractalkine (172). Furthermore, fractalkine-receptor positive leukocytes were observed in renal biopsies of patients with HUS.

In conclusion, our data show that Stx can increase the number of firmly adhering leukocytes to endothelial cells, which can be partially blocked by removal of HS. These findings strongly suggest a role for endothelial HS in the inflammatory process in the renal vasculature during HUS. However, it can not explain the preferential damage of glomerular endothelial cells observed in HUS patients.

As possible therapeutic options for future treatment, the use of N-desulfated heparin, which inhibits leukocyte adhesion and transmigration with low anticoagulant activity, or blocking of chemokine-receptors can be considered (173, 174).

In this work we have examined in more detail the effect of a subtoxic dose of Stx on the transcription and translation in Stx-sensitive cells.

Chapter 3.4

Stimulation of pro-inflammatory genes by a subtoxic dose of shiga-like toxin in primary human endothelial cells; uncoupling of transcriptional and translational effects

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Submitted

Abstract

Shiga-like toxin (Stx) is produced by *Escherichia coli* bacteria. It may damage the human renal endothelium and result in the haemolytic-uraemic syndrome. Endothelial cells internalise the toxin which destructs ribosomes, leading towards inhibition of protein synthesis. Recently, data is emerging that Stx can also stimulate cells to produce pro-inflammatory proteins. We have studied the effect of a subtoxic dose of Stx1 (without inhibition of the overall protein synthesis) on the protein synthesis of endothelial cells.

Human umbilical venous endothelial cells were incubated with a subtoxic dose of Stx1 or a positive control (TNFα or LPS). Different techniques (RT-PCR, ELISA, FACS-analysis, Western Blot) were used to analyse its effect on protein synthesis.

Stx1 was capable of increasing the mRNA of ICAM-1 and IL-8. This did not result in increased expression of ICAM-1 on the cell surface. However, the intracellular level of ICAM-1 increased slightly. But it did not approach the effect of LPS, although the amount of mRNA was equally up-regulated.

This indicates that a subtoxic dose of Stx1 may stimulate primary endothelial cells into protein synthesis with adequate transcription. Since only a small increase of intracellular ICAM-1 is noticeable, an effect of Stx1 on posttranslational modifications can not be excluded.

Introduction

The cytotoxic effect of Shiga-like toxin (Stx) has been well-described (14). Its damage of the ribosomal RNA in the endothelial cells of the renal glomeruli and arterioles is one of the hallmark-features of the pathogenesis of the haemolytic-uraemic syndrome (HUS). After binding to its specific glycosphingolipid receptor (globotriaosyl-receptor, Gb3, CD77), the toxin is internalised and transported in a retrograde direction to the endoplasmic reticulum (175). By means of this pathway, Stx is subjected to cleavage into its binding (B-) and the enzymatically active A-subunit (14). The A-subunit can leave the endoplasmic reticulum towards the cytoplasm by 'mis-using' the cellular retro-translocation machinery (67). Subsequently, the toxin reaches its target, the ribosome, which leads to disabling the translation from mRNA to proteins.

However, Gb3-receptors have also been detected on cells which are insensitive to the toxic effect of Stx. For example, the protein synthesis is not decreased after incubation with Stx in peripheral blood monocytes, cell lines for monocytes/macrophages and intestinal epithelial cells (52, 53, 176). It has been demonstrated that these cells direct the toxin towards the lysosome where it becomes degraded without damaging the ribosomes (54). During this pathway, cells are being activated resulting in the production of cytokines and chemokines (52, 55). In contrast to damaging the protein synthesis, there is a stimulating effect. This indicates that Stx seems to have a dual effect in different cell types, with the final effect depending on the amount of Stx reaching the ribosome. Tesh and his co-workers have studied this activating mechanism of Stx on monocytes/macrophages. They have described in increase of activity of transcription factors NF-κB and AP-1, and also a higher level of phosphorylation of intracellular kinases JNK1 and -2 and P38 (56, 177).

A question arising is whether also Stx-sensitive cells become primarily activated after internalising the toxin. When the gene-expression of human umbilical endothelial cells (HUVECs) incubated with a subtoxic dose of Stx was studied, an up-regulation of transcripts could be detected (149). These were predominantly transcripts of cyto- and chemokines.

In this work we have examined in more detail the effect of a subtoxic dose of Stx on the transcription and translation in Stx-sensitive cells. All experiments were performed in HUVECs. As we want to evaluate the effect of Stx on transcription factor NF-κB, cell adhesion molecule ICAM-1 and chemokine IL-8 were studied. The mRNA level of ICAM-1 and IL-8 was determined. Furthermore, the expression of ICAM-1 was determined both on the cell surface and intracellularly.

Materials & Methods

Culture and incubation of endothelial cells HUVECs were harvested after collagenase (Worthington: NJ, USA) treatment of human umbilical cords. HUVECs from 16 donors in passage 1-3 were used. Cells were grown confluent on tissue culture plastic plates coated with 1% porcine gelatine (Fluka: Neu-Ulm, Germany). Medium (M199; Gibco: Paisley, UK) with addition of 10% human serum (Cambrex: Walkersville, USA), 10% newborn calf serum (Gibco: Paisley, UK), 1% 200 mM L-glutamine (MP Biomedicals; Eschwege, Germany), 1% penicillin-streptomycine (Gibco: Paisley, UK), growth factor (extracted from bovine brains) and 0.1% heparin (Leo Pharma BV: Breda The Netherlands) was refreshed every two days.

Cells were stimulated with different subtoxic concentrations Stx1 (10 nM, 1 nM and 50 pM; Stx1 was kindly provided by Dr. M. Karmali, Toronto, Canada). Stx1 was endotoxin-free as determined with a Limulus-assay. Cells stayed viable during this stimulation, as was tested with 3H-leucine incorporation. Other used stimuli are TNFα (10 ng/ml; Roche: Woerden, The Netherlands) and LPS (1 ug/ml; Sigma-Aldrich: Zwijndrecht, The Netherlands). These stimuli are known to have a stimulatory effect on endothelial cells. Stimuli were dissolved in M199 + 20% fetal calf serum (FCS; Greiner Bio-One; Kremsmunster, Austria). Before adding the stimuli, cells were rinsed with M199.

RT-PCR for ICAM-1 and IL-8

RNA from HUVECs was isolated using the RNeasy kit (Qiagen Benelux BV; Venlo, The Netherlands) according to the manufacturer. HUVECs were incubated during 24 hours. To remove genomic DNA, the RNA samples were treated with 1u DNase (Invitrogen; Breda, The Netherlands) per 5 ml RNA for 15 min at RT. The reaction was stopped by adding 1 ml 25 mM EDTA and incubated at 65˚C for 10 min. Reverse Transcriptase superscript III (Invitrogen) was used to transcribe the RNA into cDNA (5 min. 25ºC, 60 min 37˚C and 10 min 94˚C). The reaction mixture was prepared with 2 ml 0.1 M DTT, 0.6 ml 12.5 mM dNTP's, 0.4 ml Oligo dT, 0.5 ul 40u/ml RNAsin and 0.5 ml RTsuperscript III (Invitrogen) diluted in 5x reaction buffer. cDNA was diluted 1/50 and stored at -20˚C. All primers were synthesized by Isogen lifesciences (Maarsen, the Netherlands). cDNA levels were determined using SYBR green fluorescence with the Abi-Prism 7000 (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Expression levels were evaluated as the number of cycle difference from glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Data is represented as times increase compared to unstimulated cells.

Cell-ELISA

HUVECs were grown confluent on 96-wells culture plates (Corning; Schiphol-Rijk, The Netherlands) coated with 1% gelatine (n=10). After incubation of the cells with Stx1, LPS or TNFα for various time points, the stimuli were removed and cells were rinsed with pre-warmed M199. Then cells were fixed with cold glutaraldehyde for 10 min at room temperature. After fixing, the cells were washed 3 times with phosphate-buffered saline (PBS). Cells were then incubated with primary antibodies against cell adhesion molecules (ICAM-1, kindly provided by Dr Westphal, Nijmegen, the Netherlands; VCAM, Dako, Glostrup, Denmark; E-selectin, Santa Cruz, Heidelberg, Germany) during one hour at 37˚C dissolved in M199+8% FCS. After washing 5 times with PBS/Tween 0.05%, the second antibody was exposed to the cells (goat-anti-mouse peroxidase; Dako). This was performed in a dilution of 1:1000 for 30 min at 37˚C. Ortho-phenylenediamine was added as a substrate for 30 min. 2M H_2SO_4 was added to arrest the reaction. Extinction was determined with a plate-reader at 492 nm. Data are presented as percentage of unstimulated cells. Also, experiments were performed to determine the presence of interleukin-8 in the supernatant. However, the positive control did not result in a measurable level (data not shown).

FACS

HUVECs were grown confluent on 24-wells culture plates (Corning) coated with 1% gelatine (n=3). After incubation of the cells with LPS, Stx1 in M199 with 20% FCS during 4, 14, 24 and 48 hr, the cells were rinsed with PBS and incubated with a FITC-labelled primary antibody against ICAM-1 (Dako). This was performed in M199 with 8% FCS for 60 minutes at 4˚C. After this incubation, cells were washed with cold PBS and detached. After detachment, the cells were fixated with 0.5% paraformaldehyde. Cells were analyzed with a FACS analyzer. Data is presented as percentage positive cells.

Western blot

HUVECs were grown confluent on 6-wells culture plates (Corning) coated with 1% gelatine. After incubation of the cells with stimuli for 24, 48 and 72 hr, cells were harvested and immediately centrifuged at 200 xg at -4˚C. Then cells were dissolved in a RIPA-buffer mixed with protease-inhibitors (30 µl/ml aprotinin: Sigma-Aldrich; 10 mg/ml PMSF: Sigma-Aldrich; 10 µl/ml 100 mM sodium orthovanadate). After 30 min on ice followed by centrifugation, supernatants were stored at -20˚C. From cell lysates 1 µg protein was run on 7.5% SDS-polyacrylamide gels by electrophoresis and then electrophoretically transferred to a nitrocellulose membrane. After blocking the membrane with PBS/Tween 0.5%/5% Protifar Plus (Nutricia: Zoetermeer, The Netherlands), it was incubated with a mouse monoclonal antibody against ICAM-1 (Santa Cruz: Heidelberg, Germany). An antibody against GAPDH was used as a loading control. A peroxidase-labelled antibody (Dako) was used as a secondary antibody before staining the membrane.

Results

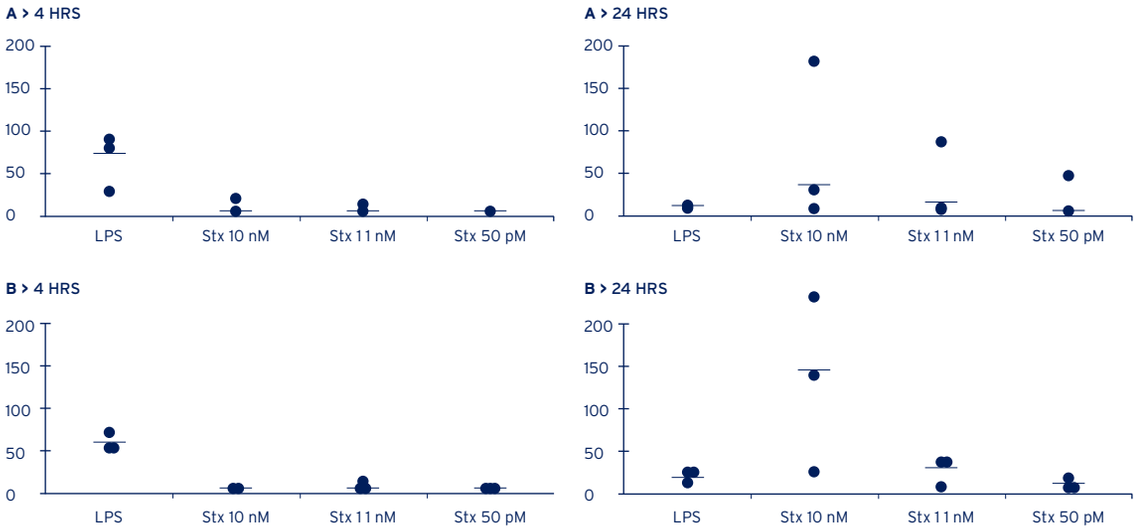
Stx1 is not able to inhibit protein synthesis of endothelial cells without pre-incubation with TNFα

When HUVECs were incubated with Stx1 10 nM or 1 nM, no inhibition of the protein synthesis could be detected with 3H-leucine incorporation. Only after pre-incubation with TNFα, which increases the Gb3-receptor for Stx1, Stx1 was able to decrease the protein synthesis of HUVECs (178).

The level of mRNA increases after incubation with Stx1

Figure 1 represents the data concerning the levels of mRNA for ICAM-1 and IL-8 after incubation with different concentrations of Stx1, or LPS which is the positive control (n= 3 HUVECs donors). LPS is able to up-regulate the level of mRNA of both proteins after 4 hours of incubation. At this time-point, no effect of Stx can be detected. However, after an incubation time of 24 hours, it is evident that also Stx increases the mRNA of ICAM-1 and IL-8, while the effect of LPS has already diminished.

Figure 1 mRNA of ICAM-1 and IL-8 after incubation with LPS or Stx1



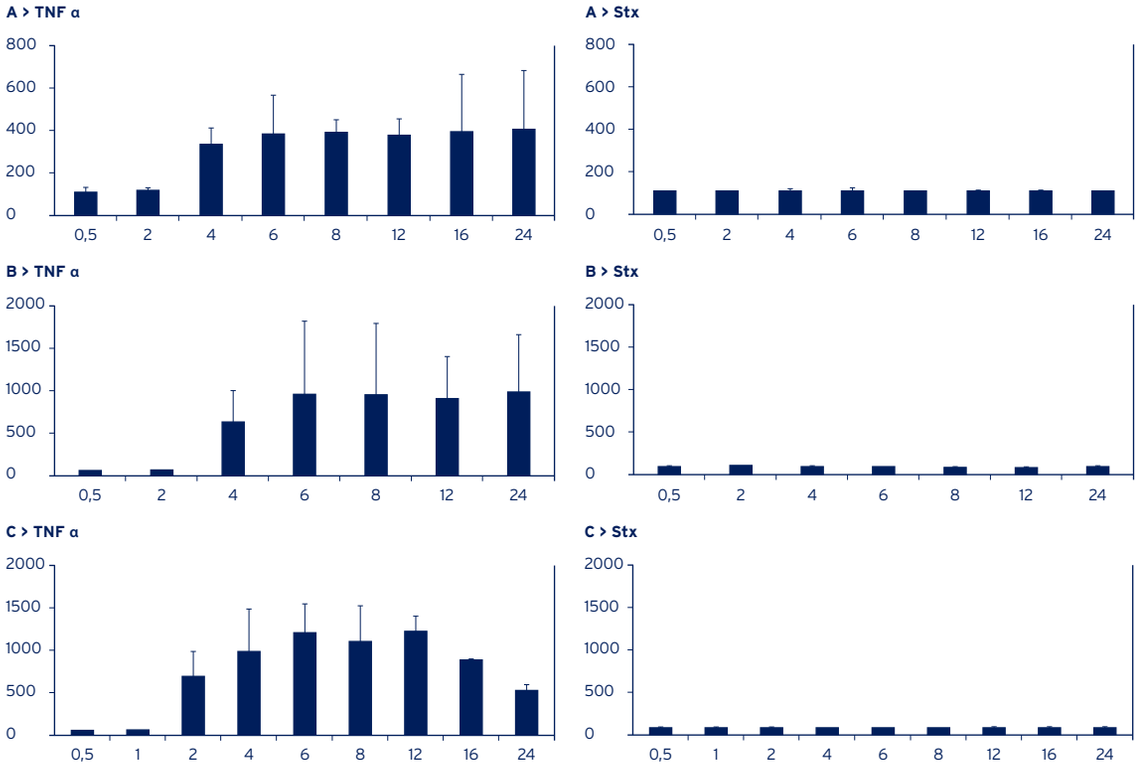
Vertical:
A: Times increase mRNA ICAM-1
B: Times increase mRNA IL-8
Horizontal: Stimuli

HUVECs (n=3) were incubated with LPS or different concentrations of Stx1 (10 nM, 1 nM and 50 pM) .The level of mRNA from ICAM-1 (A) and IL-8 (B) was measured. LPS increases the level of both ICAM-1 and IL-8 after 4 hours. Stx1 10 nM also increases mRNA of ICAM-1 and IL-8, but after 24 hours.

The expression of ICAM-1 on the cell surface does not change after incubation with Stx

To study the effect of the up-regulation of mRNA, the expression of ICAM-1 on the cell surface of HUVECs was determined (n=3). Also, the expression of cell adhesion molecules VCAM and E-selectin were measured. Figure 2 demonstrates that TNFα is capable of increasing the expression of all three different cell adhesion molecules after 4 hours. However, incubation with Stx1 does not alter the amount of expressed ICAM-1, VCAM and E-selectin at any time point. Experiments with different concentrations of Stx1 (10 nM, 1 nM, 100 pM, 10 pM, 1 pM, 100 fM, 10 fM) at different time points showed no dosage effect of Stx1 (data not shown). Furthermore, four donors of HUVECs were incubated with Stx1 10 nM for 48 hours, since the level of mRNA after incubation with Stx increased after 24 hours. Again, no effect of Stx1 could be detected on the expression of ICAM-1 (figure 3).
Another technique, FACS-analysis, was performed to evaluate these conflicting results (figure 4). These results confirm that there is no clear effect of Stx after 48 hours of incubation, whereas the effect of LPS is obvious.

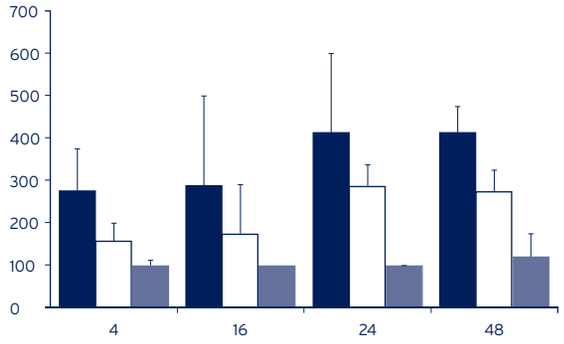
Figure 2 Effect of Stx on expression of ICAM-1, VCAM and E-selectin



Vertical:
A: % ICAM of control
B: % VCAM of control
C: % E-selection of control
Horizontal: Incubation (hours)

HUVECs (n=3) were incubated with TNFα (10 ng/ml) or Stx1 (10 nM) during various time points. The expression of ICAM, VCAM and E-selectin was determined with cell-ELISA. TNFα is able to increase the expression of all three cell adhesion molecules. Stx1 does not have an effect.

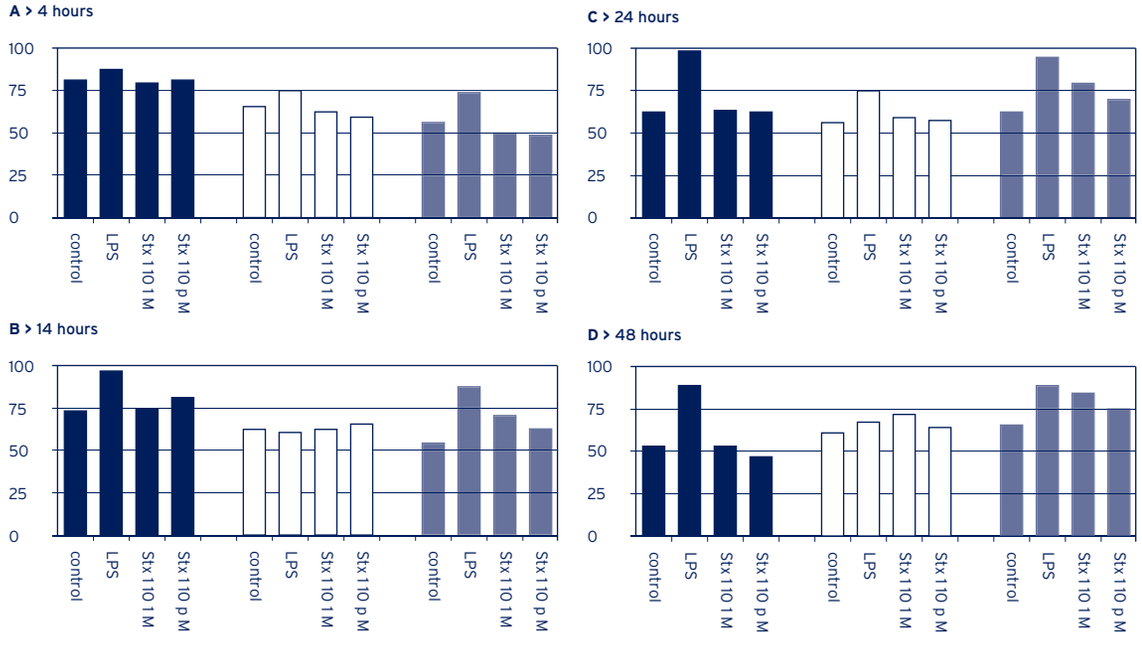
Figure 3 Effect of a prolonged incubation with Stx1 on the expression of ICAM-1



Vertical: % extinction of control
Horizontal: Incubation (hours)

HUVECs (n=4) were incubated with LPS, TNFα or Stx1 10 nM for 48 hours. Again, no up-regulation of ICAM-1 by Stx1 could be detected. LPS and TNFα are able to increase the expression of ICAM-1. The error-bar represents the standard-deviation.

Figure 4 FACS analysis of ICAM-1 expression on HUVECs



■ Donor 1
□ Donor 2
■ Donor 3

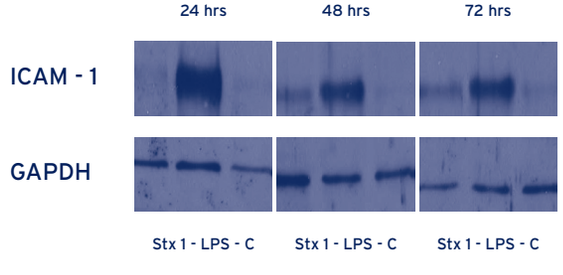
Horizontal: % positive cells

HUVECs (n=3) were incubated for 4 (panel A), 14 (panel B), 24 (panel C) and 48 hours (panel D) with Stx1 (10 nM and 10 pM) or LPS (1 ug/ml). The expression of ICAM-1 was analysed with FACS. LPS can up-regulate the amount of expressed ICAM-1. Stx1 does not have a clear effect.

The intracellular level of ICAM-1 is increased at 72 hours of incubation with Stx

To analyze the possibility that Stx up-regulates ICAM-1 without an expression on the cell surface, we have performed Western blots. Whole cell lysates were used. Figure 5 demonstrates a blot representative of the performed experiments (n=3). After 24 hours of incubation with LPS there is an evident up-regulation of ICAM-1. However, a small increase of ICAM-1 can be detected at 72 hours of incubation with Stx1.

Figure 5 Western blot of ICAM-1



HUVECs (n=3) were incubated with LPS and Stx1 for 24, 48 and 72 hours. GAPDH was used as a loading control. Whereas LPS increases ICAM-1 already after 24 hours, Stx1 gives a small raise of ICAM-1 after 72 hours of incubation. This blot is representative of the performed experiments.

Discussion

A subtoxic dose of Stx1 was able to induce mRNA for ICAM-1 and IL 8 in HUVECs, although at a later time than observed after LPS. In bovine aortic endothelial cells a subtoxic dose of both Stx1 and Stx2 induced an increase of pre-proET-mRNA levels which was evident as early as 4 hours, with maximal effect at 12-24 hours. Nuclear transcription and actinomycin D chase experiments indicated that Stx increased the half-life of pre-proET-mRNA transcripts 2.5 fold without an increase in synthesis rate (179). Matussek *et al* performed micro-array analysis and found that the main changes elicited by sub-inhibitory concentrations of Stx concerned changes in gene expression mainly for genes encoding chemokines and cytokines (149). The gene expression of IL-8 (at 4 and 24 hours) by both Stx1 and Stx2 and of ICAM-1 (at 24 hours by Stx1 and at 4 and 24 hours after Stx2) was increased. The transcription factor NFκB is involved in the stimulation of mRNA for IL-8 and MCP in human endothelial cells (170).
Newly synthesized mRNA passes through the nuclear membrane into the cytoplasm, where it becomes associated with the ribosomes for polypeptide synthesis. The changes in the level of mRNA in our experiments were not reflected in corresponding protein changes. Only 72 hours after stimulation, a small increase in ICAM-1 concentration was observed by Western blotting. Apparently, Stx1 is impairing the translation process, although the overall synthesis of protein was intact as proven by the leucine incorporation assay. The apparent discrepancy between mRNA and protein levels might be explained by post-transcriptional buffering mechanisms such as RNA interference effects. Such options should be addressed in future more detailed studies (180). The negligible increase in the surface expression of ICAM-1 is expected based on the data of the Western blotting. The influence of post-translational modification on the cell membrane trafficking can also not be excluded (181). The low mRNA concentration of ICAM-1 and interleukin-8 in the control situation precluded the evaluation of changes in mRNA degradation after transcriptional blockade.
These results differ from those observed in the monocyte and macrophage. In a human monocytic line, after 12 hours of stimulation with a subtoxic dose of Stx TNFα mRNA was raised together with an increase in TNFα production (177). In the macrophage, TNFα mRNA peaked at 6 hours, while TNFα production was optimal at 12-15 hours (182).
For the interpretation of the action of Stx in HUS, not only the effect of a toxic dose, but also the effect of a subtoxic dose has to be considered.

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Chapter 4
Non-Shiga-like toxin haemolytic uraemic syndrome

In this report a familial hetero-zygous mutation of IF is described in a patient with atypical HUS.

Chapter 4.1
A missense mutation in factor I (IF) predisposes to atypical haemolytic uraemic syndrome

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Abstract

A genetic predisposition involving complement regulatory genes has become evident in some patients with atypical HUS. In this paper, a patient with a heterozygous missense mutation in factor I (IF) is described. Although the serum level of IF was normal, a mild functional defect in the alternative pathway of complement could be demonstrated in the affected members of the family. After an episode of atypical HUS, chronic renal insufficiency started at the age of 15 months. Recurrence of HUS, with loss of the renal transplant, occurred twice in this patient. The recurrence of HUS in the graft was not reflected by haematological abnormalities (haemolysis, thrombocytopenia). One additional transplant was lost due to arterial thrombosis of the renal artery. This report confirms the gloomy outcome of renal transplants in patients with a IF deficiency. New therapies should be evaluated in these patients.

Introduction

Atypical haemolytic uraemic syndrome (HUS) should be separated from typical or shiga-like toxin induced HUS. In typical HUS, the classical features (haemolytic anaemia, thrombocytopenia and renal failure) are mostly preceded by diarrhoea. The atypical forms of HUS can be sporadic, familial and relapsing. A genetic predisposition involving complement regulatory genes has become evident in some patients with atypical HUS (reviewed in reference 183). Mutations in the plasma complement regulator factor H (HF1) and, the transmembrane complement regulator membrane cofactor protein (MCP) and patients with anti-HF1 auto-antibodies have been described as causes of atypical HUS (183). Both proteins are acting as cofactors for complement IF. This is a soluble regulatory serine protease of the complement system. It mediates the cleavage of the α-chain of C3b leading to the formation of iC3b, followed by its further degradation into C3dg and C3c. Furthermore, IF cleaves C4b to yield C4c and C4d. This will lead to an inhibition of the complement system and acts to protect host surfaces against complement activation. Also CR1 receptor (CD35) can function as a cofactor for IF.

The gene encoding for IF is located on chromosome 4q25. It spans 63 KB and comprises 13 exons. It has a molecular weight of 88.800 Da and is composed of two polypeptide chains (MW 50.000 and 38.000 Da), which are linked by disulfide bounds. The different structural protein domains of IF are well established. The first exon is a leader sequence, which is not present in the mature protein. The second exon encodes a module only found in complement C6 and C7, the so-called IF membrane attack complex module. A scavenger receptor domain is encoded by the third and fourth exon. The fifth and sixth exon each encode a low-density lipoprotein receptor module. Two small exons separate the first six exons from the last five that encode the serine protease domain of IF, forming the light chain (184). This serine protease domain is occupied with the cleavage of the above mentioned complement factors. In this report a familial heterozygous mutation of IF is described in a patient with atypical HUS. This patient showed a normal concentration of IF in serum. However, the mutation had dire consequences for the transplanted kidney. Data about the other members of the family are provided. Finally, new therapeutic possibilities for patients with defective or deficient IF are proposed.

Case report

Our patient is the third child in a non-consanguineous family with three children. The pregnancy and neonatal period were undisturbed. A relative in the paternal lineage (daughter of his father's sister) died of HUS at the age of 25. The diagnosis was made at autopsy, while she was abroad.

At the age of 15 months the patient was referred to our paediatric nephrology ward by the local paediatrician. One week before admission he developed a sore throat, otitis, coughing and a general malaise. Diarrhoea was not present. The child became gradually pale and icteric and started vomiting. Laboratory values demonstrated haemolytic anaemia with burr-cells and renal dysfunction. The diagnosis of HUS was made. Initially, the renal function recovered, followed by a second phase of renal impairment ending in permanent damage. Peritoneal dialysis had to be started. Six months later he had a relapse of HUS, accompanied by diarrhoea, which remitted spontaneously. Severe hypertension required bilateral nephrectomy.

The first renal transplantation, with a cadaver kidney, occurred when the patient was 3,5 years old. Prednisone and cyclosporine were the immunosuppressive treatment modalities at that time. After a delayed start of the graft function, his serum creatinine became normal. Five months after renal transplantation, a rise in serum creatinine (200-250 µmol/l) was noted. Renal biopsy demonstrated in some glomeruli swollen endothelial cells with infiltration of monocytes and granulocytes. The arterioles demonstrated swollen endothelium. Some diffuse fibrosis, edema and influx of monocytes were found in the interstitium. Some mesangial C3 staining was observed. Peritubular capillaries did not stain for C4d. Recurrence of HUS was suspected. The rejection was classified as IA. On the day preceding the biopsy thrombocytopenia, fragmentocytes and decreasing haemoglobin were absent. High dose prednisone was given and cyclosporine was switched to azathioprine. Uncontrollable hypertension (205/110 mmHg) with further deterioration of the renal function prompted transplantectomy, eight months after transplantation. The conclusion of the pathology report of the renal transplant was chronic vascular rejection with late and recent signs of intravascular coagulation probably due to recurrence of HUS.

At the age of seven years, he received a second renal transplant. He received cyclosporine and prednisone as immune-suppressive treatment together with thrombosis prophylaxis (low molecular weight heparins). A renal arterial thrombosis occurred at the first day and led to a transplantectomy.

At the age of 10 years he received a third renal transplant with a cadaver kidney, under the usually immunosuppressive and anti-coagulant treatment (cyclosporine, prednisone, low molecular weight heparins). Diuresis occurred rapidly and serum creatinine declined. However, at the sixth day postoperatively serum creatinine rose till 230 µmol/l. Due to a bleeding tendency, no biopsy could be performed. Despite treatment with anti-thymocyte immunoglobulin (ATG) followed by high doses of prednisone, renal function further deteriorated. Haemodialysis treatment had to be started again and transplantectomy was performed six weeks after transplantation. During the post-transplantation period, a decreasing number of platelets and haemoglobin was not observed. Pathology report of this third renal transplant showed significant fibrosis and proliferation of the intima of the arterioles with infiltration of monocytes and some granulocytes. Most of the glomeruli were ischaemic. Others show swollen endothelial cells with monocytes and granulocytes. In some arterioles thrombosis was seen. Focal staining for C4d of peritubular capillaries was present. Swollen podocytes and broadening of the subendothelial space with fluffy material were observed by electron microscopy. Extensive fibrin and C3 deposition were present along the glomerular capillary walls. Based on these pathological findings, the diagnosis of HUS was made.

Subjects & Methods

Molecular genetic studies

Genomic DNA from our patient, his two sisters and his parents was prepared from whole blood using the method first described by Miller *et al* (183). The IF gene was amplified in 11 fragments (exon 1, 2, 3, 4, 5-6, 7, 8, 9-10, 11, 12 and 13) by the polymerase chain reaction (PCR). Fragments included both DNA sequences of the individual exons, the splice donor and splice acceptor sites. The sequences of the primers and the reaction conditions are available upon request. The amplimers thus obtained were subjected to DNA sequence analysis of the coding and non-coding strands (NM_000204). PCR products were purified with alkaline phosphatase and exonuclease (Amersham, The Netherlands). Subsequently a sequence reaction was performed using the Ready reaction sequence mix (Applied Biosystems). After precipitation the fragments were dissolved in Hi-Ditm Formamide (Applied Biosystems) and sequenced by the ABI Prism 3130 Genetic Analyzer. Sequence analyses were performed using Sequencer 4.2 software. Also, all exons of the gene encoding for MCP and exon 23 of the HF1 gene were subjected to DNA analyses.

Complement assays

Serum levels of C3 and C4 were measured by nephelometry. C3d was measured by ELISA after a precipitation step with 22% PEG 6000. Radial immunodiffusion was performed to determine the level of IF. For this measurement, a goat antibody against human IF was used (produced by the Department of Nephrology, Leiden, The Netherlands).

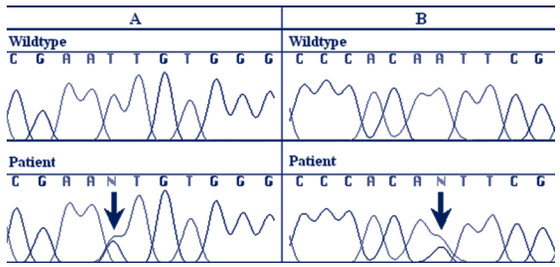
The functional analysis of the alternative pathway, as well as the classical pathway was measured with ELISA (COMPL300 Total Complement Functional Screen Kit, Euro-Diagnostica, Arnhem, The Netherlands). In short, wells were coated with either lipopolysaccharide (alternative pathway) or IgM (classical pathway) and patient serum was added for binding and activation of the specific routes. The formed end product of the complement cascade, C5b-9, was determined with an alkaline phosphatase-conjugated antihuman C5b-9 (184). The classical and alternative pathways were also assessed by the haemolytic assay (184). All assays were performed in serum from the patient and his family members.

Results

After screening the genomic DNA encoding for IF, one heterozygous 1019T > C mutation in the factor I gene was observed in our patient with atypical relapsing HUS (figure 1). This missense mutation (Ile322Thr) is located at the start of the serine protease domain (figure 2). Isoleucine is the first amino acid of a structurally conserved region of the catalytically active domain (187). The 1019 T > C mutation was absent in 112 Dutch control alleles. The patient inherited the mutation via the paternal allele. The mutation was also present in the two sisters. No mutations were found in the genes encoding MCP or HF1.

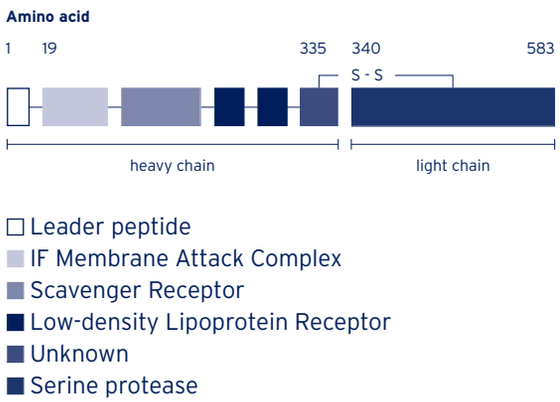
To investigate whether this mutation had an effect on the level and activity of the protein, a quantitative assay of IF in combination with a functional analysis of the alternative and classical pathway of complement was performed. The serum level of IF and HF1 were normal in the patient and his father. The alternative pathway of the complement system tested by ELISA or haemolysis had in the affected members of the family a slightly decreased or low normal functional activity, while the activity of the classical pathway remained normal. Only in the reported patient an increased serum concentration of C3d was found. Serum C3 was slightly lowered or in the low normal range in the affected members of the family (table 1).

Figure 1 Mutation in factor I



(A) Forward sequence of wild type and patient. In the patient, there is a T (ATT for isoleucine) to C (ACT for threonine) transition at position 1019, which results in an amino acid change in the serine protease domain of factor I. (B) Reverse sequence of wild type and patient at the region of the mutation. The mutation is indicated with an arrow.

Figure 2 Schematic representation of the domain structure of factor I



IF is composed of a heavy (amino acids 19-335) and a light (amino acids 340-583) chain, which are linked by a disulfide bound. The leader peptide (amino acids 1-18) and the cleavage site (amino acids 336-339) are lost from the mature protein. The mutation found in the patient is indicated with an arrow. It is detected in the first amino acid of a structurally conserved region of the catalytically active domain.

Table 1 Analysis of the complement system

	Alternative pathway		Classical pathway		C3	C4	C3d	IF	Factor H
	ELISA	Haemolytic Assay	ELISA	Haemolytic Assay					
References	> 39%	> 67 - 133%	> 74%	69 - 129%	700 - 1900 mg/l	100 - 400 mg/l	< 3,3%	186 ± 84.6 aU/ml	350 ± 203.4 aU/ml
Patient	33 26 31 22 31	66 64	90	95	607 581 550 485 561	288 286	6.02 5.31 5.50 4.85 5.61	153	300
Father	30 32	58 62	91	79	629 642	221 212	1.53 1.17	190	392
Sister 1	32 33	65 74	ND	86	771 765	163 156	1.58 1.53	ND	ND
Sister 2	28 57	56 70	ND	74	636 753	158 173	1.23 1.24	ND	ND
Mother	97	81	ND	114	989	322	1.17	ND	ND

ND: not determined

Discussion

A heterozygous mutation in IF, a regulatory protein of the complement system, was detected in our patient, his father, and his two sisters. This mutation resulted in an amino acid change in the serine protease domain. The IF concentrations in the serum of our patient and his father were found normal. The antigenic C3 levels were slightly decreased.

In all affected members of the family the functional activity of the alternative pathway was at least once lowered, while the activity of the classical pathway remained normal. Heterozygous persons in families with FI deficiency had a normal activity of the classical pathway (188). Further study is required to explain the discrepancy in functional activity in both pathways. The increased concentration of C3d in our patient is due to his disturbed renal function and some stimulating effects of the haemodialysis (189, 190).

The homozygous deficiency of IF is well studied. It results in an increased turnover of C3 and thereby in a defect in opsonification. The deficiency is clinically associated with increased susceptibility to severe infections. Particularly infections with encapsulated bacteria such as *Streptococcus pneumoniae* and *Neisserial* bacteria can be found (191). Also immune complex- mediated diseases due to defective immune complex clearances are associated with IF deficiency (192).

Fremeaux-Bacchi *et al*, Kavanagh *et al*, Esparza-Gordillo *et al* and Capioli *et al* have recently described heterozygous mutations of FI that predispose to the development of HUS (86, 94, 95, 104). Striking was the first occurrence of the disease at adult age, which was frequently observed. Pregnancy could have a favourable role. Even when the first symptoms of HUS appear at adult age, a defect in the regulatory pathway should be considered.

In contrast to patients who have a mutation in MCP, the results of transplantation in HUS patients due to a heterozygous IF-mutation are gloomy. Patients with mutations in MCP are not prone to a recurrence after transplantation. This is due to the fact that normal MCP will be present in the renal allograft, being a transmembrane protein. Considering mutations in IF, one of the patients reported by Fremeaux-Bacchi *et al* had a recurrence of HUS in two transplants. The first patient of Kavanagh *et al* had initially an excellent renal function after transplantation, but a recurrence and deterioration of the function occurred after two months. The second patient had a recurrence of HUS, which was noted twenty months after the transplantation. The sister of this second patient had two transplants that failed early. In one transplanted patient, reported by Caprioli *et al*, two grafts were lost because of the recurrence of HUS within the first year after transplantation. Our patient had a possible recurrence after the first graft, and a proven recurrence after the third graft.

Can transplantation still be recommended in patients with HUS due to a heterozygous mutation in IF? Recurrence of HUS can be expected during periods of complement activation after renal transplants. Dangerous predictable situations are ischaemia/reperfusion early after transplantation and during acute rejection (193, 194).

New therapeutic possibilities should be considered. The effect of treatment with plasma in patients with deficient IF has been studied. The half-life of IF is 24-45 hours, but with a more pronounced effect on the C3 and C4 level (195). After a plasma infusion (20 ml/kg) in our patient, the functional activity of the alternative pathway remained above 40% during at least three days (the activity before the infusion was 26%). Another possibility is treatment with Eculizimab, a humanized antibody that specifically targets the complement protein C5 and prevents its cleavage. Complement inhibition at the C5 level blocks the formation of C5b-9 while preserving early components critical for the clearance of bacteria and immune-complexes (196). Experimental work in mice is encouraging. Prevention of C5 activation ameliorated spontaneous and experimental glomerulonephritis in factor H deficient mice (197). The availability of recombinant IF will be the ultimate solution.

New treatment protocols for patients with deficient or defective IF are unavoidable in order to improve the outcome of renal transplantations in these patients. Renal transplantation is prone to failure in patients with a heterozygous mutation in IF.

Chapter 5
General discussion & future perspectives

The purpose of this thesis was to identify ingredients that contribute to the development of this multifactorial disease.

The pathogenesis of HUS involves elements of many different systems of the human body which maintain its homeostasis. Examples are the coagulation cascade, and the innate immunity with the complement system. All these different parts result in a complex disease mechanism with many interactions between the involved systems. A complete picture of the pathogenesis is lacking. And last but not least, also the individual genetic profile will contribute to this complex disease. Although researchers over the years revealed small parts to provide a better understanding, the pathogenesis is still 'a secret recipe'.
The purpose of this thesis was to identify ingredients that contribute to the development of this multi-factorial disease. Since specific treatment is still missing, a better understanding of the disease mechanism will ultimately lead to new treatment strategies.

5.1 The toxin

An important question concerning HUS is it Stx is still the central factor in HUS? Especially since Stx was never detected in serum of patients with HUS. Why are we so interested in this protein? The presence of only endotoxin from the *Escherichia coli* bacteria, called lipopolysaccharide (LPS) will not induce HUS in experimental models of animals (198). Findings that support the key role of Stx in HUS is the detection of Stx in renal biopsies of patients with HUS and the presence of Stx-anti-bodies in the serum of patients (19, 107). However, also other *Escherichia coli*-secreted proteins can be involved in the pathogenesis. Cytolethal disten-ding toxin was identified in different strains of *Escherichia coli* and caused direct damage of DNA resulting in cell death in human endothelial cells (22, 199). The gene cluster for this protein was detected in 4.9% of E coli O157:H7 (200). This toxin also proved to have a cytotoxic effect on human glomerular endothelial cells (own preliminary data). An additional detected protein is the pore-forming hemolysin. This protein was detected in EHEC-strains which did not produce Stx, although the loss of Stx-genes before analysis remains a plausible option (23). In this work, recombinant hemolysin demonstrated to be cytotoxic in a dose- and time-dependent manner to human endothelial cells. Furthermore, hemolysin is capable of activating monocytes to release cyto-kines which increase the presence of Gb3-receptors on endothelial cells (201). However, it remains to be elucidated if hemolysin is able to reach the circulation and induce a systemic effect. Paton *et al* discovered a new AB5 toxin called subtilase cytotoxin (24). This toxin is capable of cleavage of an essential endoplasmic reticulum chaperone which will induce cell death (202). This toxin was present in 32 out of 68 different Stx-producing *Escherichia coli* strains (24).
Since all three toxins demonstrated to have a direct and damaging effect on endothelial cells *in vitro*, a combined role in the pathogenesis could be possible.

5.2 Transport of Stx

Stx is the most extensively studied protein in the research of HUS. Research was especially focussed on the transport route to its main target, the renal endothelium. Interfering in this pathway will prevent Stx from damaging the kidney.
Different animal models have been tested. Siegler *et al* demonstrated a primate model of HUS (203). It was demonstrated that baboons also carry Gb3-receptors in the gut and kidney. After an intravenous injection with Stx1, they developed a biochemical picture similar to HUS. Keepers *et al* recently described a murine model in which a intraperitoneal co-injection of Stx2 and LPS is given (204). This resulted in thrombocytopenia, haemolytic anemia and renal failure. Also a canine model has been presented by Raife *et al* (205). An intravenous injection of Stx1 resulted in bloody diarrhoea and procoagulant changes similar to human. Since the animal models remain controversial concerning the presence of Gb3-receptors and their cellular sensitivity for Stx (the receptor is absent on glomerular endothelia from mice and rats), many *in vitro* studies are still performed.
More insight has been gained in understanding how Stx is transported from the intestinal tract into the circulation. Especially, since Schuller *et al* demonstrated the binding of Stx to Paneth-cells in the small intestines (33). Before, no receptor for Stx was detected in human primary intestinal epithelial cells in contrast to human cell lines.
After crossing trans-or paracellular the intestinal wall, Stx has to reach the circulation in order to damage the renal endothelium. When we consider that below the intestinal epithelial lining there is an underlying gut-associated lymphoid tissue (GALT) filled with macrophages, dendritic cells and T-lymphocytes, it is intriguing to realize that Stx can still reach the circulation (206). The effect of Stx on monocytes has been well-described, but the interaction between the toxin and dendritic cells is still to be evaluated.
Probably, the architecture of the intestinal wall is disturbed by the adherence of EHEC. This adherence combined with the passage of Stx will increase the expression of chemokine IL-8 on the cell surface (31). This results in initiation of an inflammatory response with attraction of PMN and increased permeability leading to (bloody) diarrhea. This will lead to a more pronounced access of Stx into the circulation.
It is assumed that Stx will bind specifically to a transporter in order to reach the renal endothelium. Especially since the toxin was never detected in the acute phase of HUS in serum of patients with HUS. Many blood cells have been studied, but until now none proved to be capable of this task. Our group suggested a transport-pathway by PMN (38). However, in chapter 2.2 we came to an opposite conclusion; PMN are not the transporters of Stx. Different techniques were performed to analyse a possible binding. *In vitro* experiments with both full blood and freshly isolated PMN did not result in a binding of Stx to the PMN. Also *in vivo* experiments with injection of the B-subunit from Stx in mice, demonstrated a rapid clearance from the blood without binding to PMN. When we studied Stx-binding to PMN isolated from HUS-patients, there were variable results with one patient demonstrating binding of a Stx-antibody to the PMN during active disease. However, this antibody did also bind to PMN from patients on haemodialysis in contrast to patients on peritoneal dialysis and healthy controls. This indicates that this antibody was not specific and probably binds to activated granulocytes present in patients on haemodialysis and during HUS. Furthermore, Stx was not capable of activating PMN, which suggests that no specific binding was present. Our data was recently confirmed by Flagler *et al* (207). This work demonstrated that Stx was not able to induce apoptosis of PMN. Furthermore, after addition of Stx to human whole blood, the highest concentration could be detected in the cell-free plasma fraction. Neither Stx1 nor Stx2 was found to be associated with isolated PMN. Also in their hands, the injection of Stx into mice did not result in binding to blood cells. This indicates that other possible carriers have to be explored. The binding of Stx to peripheral blood monocytes carrying a specific receptor for Stx was already demonstrated (52, 53, 54). This indicates that monocytes could be an interesting option to transport Stx to the renal endothelium. But, although monocytes contribute to the pathogenesis by producing pro-inflammatory cytokines after internalising the toxin, it could not be demonstrated

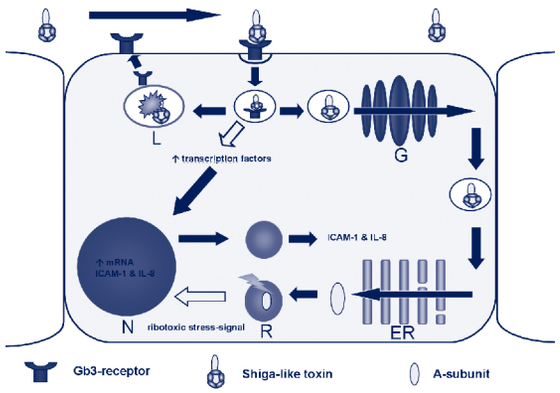
to be the transporter (see chapter 2.1). Furthermore, it shows that one needs to be cautious with the interpretation of binding experiments performed at 4˚C since binding properties change at 37˚C.
It could be hypothetically possible that Stx travels unbound in the circulation, and is directed towards Gb3-receptors present on various endothelia with its final effect dependent on the intracellular pathway of the toxin. Or should other serum proteins be tested for a role as transporter. Kimura *et al* demonstrated that protein serum amyloid P (SAP) is able to bind and neutralize Stx2 (57). This serum protein does not exist in other species. Armstrong *et al* have performed *in vivo* experiments to demonstrate a protective role of this protein (58). After intraperitoneal injection of SAP in mice, it protected them from a lethal challenge with Stx2. Also human SAP-transgenic mice were protected from Stx2. The accumulation of Stx2 in target organs (kidney and brain) was diminished in the presence of SAP in the circulation. This group also studied the presence of SAP in persons with pathogenic *Escherichia coli* infections. There was no clear difference in serum level of SAP between patients developing HUS and those without it. However, the clearly diminished accumulation in target organs indicates a possible new approach for preventing damage in HUS.

5.3 Effect of Stx toxin on endothelium

Since the retrograde transport route of Stx was described by Sandvig *et al* (14), many research groups have used the toxin as a model for this intracellular trafficking pathway. Chapter 3.1 demonstrates that this pathway is also followed by Stx in its main target; the renal endothelium and in mesangial cells. This was a missing chapter in the knowledge on HUS. The ribosomes of the renal endothelium will be attacked by Stx after retrograde transport. The increase of sensitivity of renal endothelium by cytokine TNF-α, resulted from an increased amount of Stx in the endoplasmic reticulum instead of a different transport-route. This transport-route is of crucial importance for the biological activity of Stx. Cells insensitive for the cytotoxic effect of Stx, like monocytes demonstrated a different pathway with direction of the toxin to the lysosome instead of the endoplasmic reticulum (54). The presence of the receptor in specific areas of the cell membrane (so-called detergent-resistant membranes), directs the toxin into the retrograde pathway. Probably the sensitivity of the cell type is relying on the location of the majority of Gb3-receptors. Since foreskin endothelial cells express many Gb3-receptors without cytotoxicity of Stx, it would be interesting to study the location of these receptors in the cell membrane. When a threshold of active A-subunit reaches the cytoplasma, cytotoxicity occurs. The ribosomes will be damaged and the protein synthesis will arrest, leading to cell death. But what occurs with a subtoxic dose of Stx? This will not result in an overall inhibition of the protein synthesis (132). Instead, activation of cells seems to take place. In chapters 3.2, 3.3, 3.4 subtoxic doses of Stx were used. This probably approaches the *in vivo* situation, since Stx was never found in the circulation. In chapter 3.2 a subtoxic dose of Stx was capable of releasing the content of WPbs from renal endothelial cells in flow. Two different mechanisms are possible; an increase in intracellular calcium or an increase cAMP is needed. Since Ikeda *et al* demonstrated an increase of calcium by Stx in Stx-sensitive Vero-cells (originating from the African green monkey); this could be the mechanism of release in our endothelial cells (153). Application of shear stress possibly increases the up-regulation of intracellular calcium and thereby the stimulating effect of Stx. Also in chapter 3.3, shear stress is applied to the endothelial cells. In this chapter, a subtoxic dose of Stx results in the firm adhesion of leukocytes. HSPG play a crucial role in this effect, since cleavage of HSPG reduces the amount of adherent cells. For the first time, the role of HSPG in the pathogenesis was evaluated. Since no direct effect of Stx on the HSPG could be demonstrated, an indirect effect through release and binding of chemokines to HSPG could be a plausible explanation.
But how is this activating effect initiated? How is it possible that this cytotoxic protein also has an activating effect? The results presented in chapter 3.4 demonstrate the increase of mRNA of IL-8 and ICAM-1 after incubation with a subtoxic dose of the toxin. This effect was also demonstrated by Matussek *et al* (149). But, the transcription of mRNA is not being properly followed by efficient translation, since only small amounts of protein can be detected after more than 48 hours. This indicates that instead of only disabling ribosomes, Stx is also able to activate endothelial cells, however not very efficiently. The exact mechanism is yet unclear, but up-regulation of transcription factors like NF-κB and AP-1, and also a higher level of phosphorylation of intracellular kinases JNK1 and -2 and P38 have been described in a monocytic cell line (56, 177). This may also occur in endothelial cells. Groups of Thorpe and Tesh, working with intestinal epithelial and monocytic cell lines, suggested a ribotoxic stress model occurring after incubation with a subtoxic dose of Stx (56, 117). This indicates that after damaging the ribosome, it will send a signal to stress-activated protein kinase pathways. This results in new biogenesis of ribosomes and an enhanced cytokine expression. This model may also be applicable in our endothelial cells. We hypothesize that the endothelial cell becomes activated after internalising Stx, resulting in production of mRNA-transcripts and release of WPbs content. This effect is enforced by application of shear stress. After internalising, part of the toxin is retrogradely transported to the ribosome, which is dependent on the position of the Gb3-receptor in the outer cell membrane. The other part is targeted to the lysosome and is degraded. The Gb3-receptor will be recycled and appear again on the cell surface

(208). The damaged ribosome will release a stress signal. When a subtoxic dose of Stx is applied, not every ribosome will be damaged, allowing translation of proteins. This mechanism will contribute to the pathogenesis of HUS. The proposed pathway is demonstrated in figure 1.

Figure 1 Intracellular trafficking and effects of a subtoxic dose of Stx in the renal endothelium



The filled arrows indicate a well-established transport-pathway or effect. The open arrow is hypothetical. (G: Golgi-apparatus; ER: Endoplasmic Reticulum; R: Ribosome; N: Nucleus; L: Lysosome)

One of the key question in the pathogenesis of HUS remains why specifically the renal endothelium is attacked. Possible explanations are: (a) the intracellular trafficking and sensitivity is specific in renal endothelium compared to other endothelia dependent on the position of Gb3 in the outer cell membrane; (b) there is an decreased expression of membrane-bound complement-regulatory proteins in renal endothelium resulting in an increased insertion of the membrane-attack complex thereby initiating the coagulation system; (c) an increased expression of HSPG compared to other endothelia resulting in more adhesion of leukocytes and inflammation; (d) the presence of mesangial cells which release less NO after internalising Stx resulting in vasoconstriction and thereby aggravating damage of the renal endothelium (209); (e) the presence of podocytes which release endothelin-1 after internalising Stx with effects on glomerular permeability (72).
Another unanswered question is why specifically children are prone to develop HUS? The hypothesis that paediatric renal endothelium would carry more Gb3-receptors proved not to be true (16). This indicates that other options need to be explored. Answers to the above formulated hypotheses, will direct the research in this field.

5.4 Treatment of infection-induced HUS

Garg *et al* performed a systemic review describing a total of 49 studies including 3476 patients with infection-induced HUS (210). 12% died or developed end-stage renal disease and 25% of survivors demonstrated long-term renal sequelae. Especially the occurrence of central nervous system symptoms and need for initial dialysis is associated with a worse long-term prognosis. A better treatment is still needed. Since Stx is the leading element in damaging the kidney, attempts have to be made to capture it before it reaches its goal. Different agents have been developed to bind and inactivate Stx. The first agent which was tested double-blind, randomized and placebo-controlled in children with infection-induced HUS was a synthetic Stx-binder, called Synsorb-Pk (211). Unfortunately, this product did not show any significant improvement in the course of disease. The administration was probably too late and could not influence the course of the disease. Another possibility is the use of Stx-binding enteral bacteria. Pinyon *et al* engineered a recombinant bacterium based on a non-pathogenic *Escherichia coli* strain which successfully binds Stx and protects mice from lethal doses of *Escherichia coli* O157:H7 (212). But again, these bacteria have to be given very early to prevent renal damage. Until now, different synthetic compounds with greater affinity for Stx have been developed. Human studies have to be awaited. Recently, Gu *et al* reported that heparin was able to block the adhesion of *Escherichia coli* O157:H7 to human colonic epithelial cells by blocking binding of the bacteria to immobilized epithelial beta-1 integrins (213). If this will result in therapeutic options, needs to be further investigated. Another interesting option to bind Stx is the use of monoclonal antibodies. Mukherjee *et al* developed human monoclonal antibodies which prevented Stx-cytotoxicity in both Stx-sensitive mice and piglets (214). Phase 1 trials in humans are being performed. The most important problem in these therapeutic options is the amount of renal damage already present at the moment of presentation. Initiated damage can not be restored, although progression of damage can be possibly prevented.
Raife *et al* described the possibility of prevention with lepirudin, a thrombin-blocker, which improved the clinical outcome in a canine model of HUS (203). Supportive care and prevention of further damage by optimising above described approaches remain the best possible options of treatment at this moment.

5.5 HUS caused by disorders of complement regulation

The obtained insight in the pathogenesis of this specific form of HUS is enormous. Specific mutations have been described in complement regulatory proteins like HF1, IF, MCP and BF. Most of the mutations result in a loss of function, but the latter being a gain-of-function mutation. To carry a mutation in these proteins does not always result in developing HUS. Like in chapter 4.1, a mutation in Factor I predisposed the patient to develop HUS whereas his healthy father and sisters also carry the mutation. This chapter also described that a normal level of the protein cannot exclude the involvement of this protein. Kavanagh *et al* demonstrated that the mutation described in chapter 4.1 results in a secreted protein that lack C3b and C4b cofactor activity (215).

Recently, Pickering *et al* developed the first mouse model of non-Stx HUS (216). This model provided the knowledge that heterozygous mutations in HF1 resulting in an effective plasma C3 regulation combined with a defective regulation on the renal endothelium, will lead to a spontaneous development of HUS. If there is a complete deficiency of HF1, membranoproliferative glomerulonephritis type II will occur.

Until now, in around 50% of patients with non-Stx HUS, the genetic cause of the disease is still unknown. This stimulates investigators to find other possible involved complement proteins. Also, combinations of polymorphisms need to be considered.

The knowledge of which complement protein is involved is important for optimising the treatment of patients with non-Stx HUS. Since complement factors are membrane-bound or circulating, this can predict whether kidney transplantation can be successful. Patients with mutations in membrane-bound proteins like MCP can be transplanted. However, circulating mutated complement proteins tend to initiate a relapse after transplantation. Combined liver and renal transplantation is a possibility, but remains controversial (217). Live-related donations should not take place until the underlying defect in the patient and occurrence in the donor is examined. Also patients, who develop a first occurrence of HUS after renal transplantation, should be screened for mutations in the complement control proteins.

The best therapeutic options in this type of HUS would be to add the recombinant form of the protein to patients with mutations. Since patients with HF1 is the largest and therefore most interesting group for the pharmaceutical industry, recombinant HF1 is being prepared for therapeutic treatment. This is not the case for recombinant IF. In these patients, plasmapheresis is the preferred therapeutic option. This will lead to an addition of intact IF, preventing the occurrence of HUS.

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English Summary & Nederlandse samenvattingen

Summary English

HUS consists of haemolytic anaemia, thrombocytopenia and acute renal failure. There are many different forms of which infection-induced and disorders of complement regulation are the most frequent diagnosed ones.

The purpose of this thesis was to identify new ingredients in the pathogenesis of HUS. Until now, the disease mechanism of HUS is a secret recipe. A better understanding of the underlying mechanism of disease can lead to improved treatment strategies.

Chapter 1 is an introduction to the experimental work of this thesis, with a general overview of current knowledge on HUS. The most recent knowledge on the two above mentioned forms of HUS will be presented. Concerning infection-induced HUS, the knowledge on the journey of Stx including passage from the intestinal tract into the circulation and its passage to the renal endothelium will be presented. Disorders of complement regulation can also cause HUS; the involved proteins and their mutations are also described in **chapter 1**.

Chapter 2 presents the work on the transport of Stx in the circulation. For many years now, researchers are longing to find a specific transporter to explain how Stx is specifically targeted to the renal endothelium. In **chapter 2.1**, the PMN as a possible carrier was evaluated. Although former experiments demonstrated that PMN could be the transporter for Stx in patients with HUS, new experiments resulted in the opposite. The possible interaction was studied with different techniques. It was not possible to bind Stx to human PMN *in vitro* or to PMN originating from mice *in vivo*. The binding of Stx-antibodies to PMN appeared to lack specificity. PMN from patients after haemodialysis were able to bind the antibodies. This could indicate that the antibody also binds to a specific structure on membranes of activated PMN. Using these antibodies for a diagnostic purpose in HUS is not permitted.

Chapter 2.2 presents the experiments which investigated whether the monocyte could be the carrier for Stx in the circulation. Monocytes have a specific receptor for Stx and are not sensitive for its cytotoxic effect. Instead, they become activated and produce pro-inflammatory cytokines. The work in this chapter explains how after promising first results, it was concluded that also the monocyte is not capable of transport. After a necessary change of temperature after binding Stx to monocytes and before incubation with the target cells, the toxin became released. This indicates how careful data from *in vitro* experiments performed below body temperature needs to be interpretated. When experiments were performed without any change of temperature, no transfer of Stx from monocytes to target cells could be detected

In **chapter 3** the effect of Stx on endothelial cells was evaluated. Next to cytotoxicity at a high dose, a subtoxic dose of the toxin proved to have a stimulating effect.

In **chapter 3.1** the intracellular pathway of Stx in primary glomerular endothelial and mesangial cells are evaluated. This missing knowledge is important for interpretation of the effect of Stx on its main target, the kidney. After internalising, the toxin follows the retrograde pathway in both cell types. This enables the toxin to reach and damage the ribosome. Incubation with TNF-α upregulated the receptor for the toxin (Gb3), thereby increasing the quantity of transported toxin. Not the transport pathway itself changed.

Chapter 3.2 describes the effect of Stx on WPbs. These endothelium-specific storage vesicles contain many important proteins for inflammation (IL-8) and coagulation (VWF). These evidently play a role in the pathogenesis of HUS. To approach the *in vivo* situation, shear stress was applied to the endothelial cells. When GMVEC was exposed to Stx in flow conditions, exocytosis of WPbs could be detected with confocal microscopy. Since GMVEC is the primary target for Stx, this could contribute to the pathogenesis of HUS.

In **chapter 3.3** the effect of Stx on the adhesion of leukocytes to the endothelium was evaluated. It was already described that Stx increased the amount of adherent leukocytes, but the role of HSPG in this process was yet unknown. When HSPG was cleaved from the endothelial cells after incubation with Stx, a decrease of adhering leukocytes was detected. An increase of chemokines attached to HSPG is an acceptable hypothesis.

In **chapter 3.4** the apparent stimulating effect of Stx on endothelial cells was explored. An increase

in mRNA of ICAM-1 and IL-8 could be detected. However, translation of this mRNA occurred very inefficiently. Small amounts of protein could be detected only after 72 hours. A subtoxic dose of Stx is able to both activate the cell and inhibit the translation process of new proteins.
Chapter 4.1 demonstrates a novel heterozygous mutation in IF in a patient with non-Stx HUS. Although, the serum level of IF was normal, a mild functional defect could be detected. Interestingly, two siblings and the father also carried the mutation, but did not experience any clinical problems. This indicates that the defect forces a predisposition.

HUS consists of haemolytic anemia thrombocytopenia and acute renal failure.

Samenvatting Nederlands

De naam HUS omvat een complexe multi-factoriele aandoening. Het bestaat uit hemolytische anemie, thrombocytopenie en acuut nierfalen. Er zijn verschillende vormen van HUS beschreven, waarvan infectie-gerelateerd en stoornissen in de complement-regulatie de oorzaken zijn die het meest frequent worden gediagnosticeerd.
Het doel van dit proefschrift was om nieuwe ingrediënten in de pathogenese van HUS te identificeren. Tot op de dag van vandaag is het exacte ziektemechanisme een geheim recept. Een beter begrip van het ontstaan van HUS kan leiden tot nieuwe behandelingsstrategieën. Vooral voor de infectie-gerelateerde HUS is dit belangrijk, omdat slechts ondersteunende behandeling kan worden gegeven. De oorzaak wordt nog steeds niet aangepakt.
Hoofdstuk 1 is een inleiding op het experimentele werk van dit proefschrift. Het is een algemeen overzicht van de huidige kennis over HUS. De nieuwste inzichten zullen worden beschreven. Wat betreft de infectie-gerelateerde HUS wordt de route van Stx van de darm via de circulatie naar het nierendotheel uitvoerig beschreven. Recent zijn nieuwe inzichten verworven in de kennis over het transport van Stx over de darm. Het transport van Stx in de circulatie is nog steeds onduidelijk en zal worden behandeld in dit proefschrift. Tevens zal de huidige kennis over de intracellulaire route worden beschreven. Dit leidt tot een beter begrip van de gevoeligheid van de cel voor Stx en de celbiologische aspecten. Wat betreft de HUS door stoornissen in complement-regulatie, zullen de betrokken eiwitten en de bekende mutaties worden beschreven.
Hoofdstuk 2 omvat het experimentele werk over transport van Stx in de circulatie. Al gedurende lange tijd zijn onderzoekers op zoek naar een specifieke transporter van Stx. Dit om te begrijpen hoe Stx specifiek naar het nierendotheel wordt getransporteerd. In **hoofdstuk 2.1** wordt geëvalueerd of de PMN een mogelijke transporter voor Stx is. Alhoewel eerdere experimenten aantoonden dat PMN in staat waren om Stx te transporteren, toonden de nieuwe experimenten dit niet aan. Er werd geen binding gevonden tussen Stx en humane PMN *in vitro* of PMN van muizen *in vivo*. Bovendien werd aangetoond dat er geen specifieke binding was van een Stx-antilichaam. Alhoewel er binding plaatsvond van het antilichaam aan PMN van een patiënt met acute HUS, werd ook binding gezien aan PMN van patiënten met hemodialyse. Dit betekent dat het antilichaam kan binden aan specifieke structuren op het membraan van een geactiveerde PMN. Kortom, het detecteren van Stx op PMN met behulp van antilichamen is geen goede diagnostische test voor HUS.
Hoofdstuk 2.2. presenteert experimenten waarin de monocyt als transporter voor Stx wordt bestudeerd. Monocyten hebben een specifieke receptor voor Stx en zijn niet gevoelig voor het cytotoxische effect. Ze raken zelfs geactiveerd en produceren vervolgens pro-inflammatoire cytokines. Dit hoofdstuk begint met veelbelovende data, maar uiteindelijk wordt geconcludeerd dat ook de monocyt niet in staat is om Stx te transporteren naar net nierendotheel. Na een benodigde temperatuurswisseling, liet het toxine los van de monocyt. Dit betekent dat men altijd voorzichtig moet zijn om de data van experimenten uitgevoerd onder 37˚C te implementeren naar de humane situatie. Echter, wanneer de experimenten werden uitgevoerd zonder temperatuurswisseling, werd geen transfer van Stx vanaf de monocyten naar de doelwitcellen gezien. Maar, buiten kijf staat dat monocyten een belangrijke rol spelen in de pathogenese van HUS.
In **hoofdstuk 3** wordt het effect van Stx op endotheelcelen geevalueerd. Naast cytotoxiciteit, kan een subtoxische dosis ook een stimulerend effect hebben. Na internalisatie van het toxine, wacht de endotheelcel niet passief totdat de eiwitsynthese wordt geblokkeerd. Echter, het doet een poging om zichzelf te beschermen tegen ernstige schade.
Hoofdstuk 3.1 toont de intracellulaire route van Stx in primaire glomerulaire endotheliale en mesangiale cellen. Deze ontbrekende kennis is belangrijk voor de interpretatie van het effect van het toxine op zijn belangrijkste doel, de nier. Na internalisatie van het toxine, volgt het de retrograde route. Dit stelt het toxine in staat om het cytoplasma en dus de ribosoom te bereiken. Pre-incubatie met TNF-α, wat de gevoeligheid van beide celtypes voor Stx vergroot, verhoogde de kwantiteit van getransporteerd toxine door een toename van Gb3-receptoren en niet door een aanpassing van de route. Bovendien, was

TNF-α niet in staat om de positie van Gb3 in het celmembraan te veranderen. Er was geen toename van Gb3 in de DRM, wat zou resulteren in een sterkere toxiciteit.

Hoofdstuk 3.2 beschrijft het effect van Stx op WPbs. Deze endotheel-specifieke opslagvesikels bevatten belangrijke componenten voor inflammatie (IL-8) en coagulatie (VWF). Deze eiwitten spelen een evidente rol in de pathogenese van HUS. Om de *in vivo* situatie zo goed als mogelijk te benaderen, werd 'shear stress' toegepast aan de endotheel cellen. Wanneer GMVEC werd blootgesteld aan Stx 'in flow', kon exocytose van de WPb worden gedetecteerd met behulp van confocale microscopie. HUVEC reageerde niet 'in flow', maar toonde een klein effect in statische condities. Aangezien GMVEC het primaire doel is voor Stx, kan dit effect een bijdrage leveren aan de pathogenese van HUS.

In **hoofdstuk 3.3** werd het effect van Stx op de adhesie van leukocyten aan endotheel bestudeerd. Het was reeds bekend dat Stx in staat was om de adhesie van leukocyten aan het endotheel te doen toenemen. Dit effect werd toegeschreven aan toename van cytokines. Echter, de rol van HSPG in dit proces was niet bekend. Wanneer HSPG werd verwijderd van endotheelcellen na incubatie met Stx, werd een afname van het aantal adhererende leukocyten gezien. Dit kan twee verschillende mechanismes hebben: een toename in de expressie van HSPG of een toename in chemokines gebonden aan de HSPG. Deze laatste mogelijkheid is het meest waarschijnlijk.

In **hoofdstuk 3.4** wordt het stimulerende effect van Stx op endotheelcellen nader onder de loep genomen. Een toename van mRNA van ICAM-1 en IL-8 kon worden gemeten. Echter, de translatie van dit mRNA vond zeer inefficiënt plaats. Slechts kleine hoeveelheden eiwit konden worden gemeten na meer dan 48 uur incubatie. Dit suggereert dat naast het cytotoxische effect van het toxine, een subtoxische dosis in staat is om de cel te activeren. Het is nog niet duidelijk wanneer tijdens het intracellulaire transport dit plaats vindt. Dit behoeft verder onderzoek.

Hoofdstuk 4.1 beschrijft een nieuwe heterozygote mutatie in IF in een patiënt met non-Stx HUS. Alhoewel de waarde in het serum als normaal werd beschouwd, kon een mild functioneel defect worden gedetecteerd. Het is interessant om in achting te nemen dat zowel de vader als de twee zussen ook drager zijn van de mutatie, zonder kliniek hiervan. Dit impliceert dat ook deze vorm van HUS multifactorieel is.

Dit suggereert dat naast het cytotoxische effect van het toxine, een subtoxische dosis in staat is om de cel te activeren.

Samenvatting
Voor niet-medici

Het hemolytisch-uremisch syndroom (HUS) is een nieraandoening welke vooral op jonge leeftijd kan ontstaan. Het heeft verschillende oorzaken zoals het doormaken van een infectie met een specifieke bacterie of ten gevolge van een aangeboren defect in het afweersysteem. De verschillende vormen geven wel dezelfde kenmerken: (1) een tekort aan rode bloedcellen door beschadiging, (2) een tekort aan bloedplaatjes en (3) plotseling optredende uitval van de nieren. Het is echter niet geheel duidelijk hoe deze aandoening nu ontstaat en daardoor is een goede behandeling niet mogelijk. Alleen de consequenties van HUS kunnen nu worden bestreden door middel van bijvoorbeeld transfusies van bloedproducten, nierdialyse en in ernstige gevallen een niertransplantatie. Om dit te kunnen verbeteren en de ziekte directer aan te kunnen pakken voordat de nier wordt beschadigt, moet meer onderzoek worden gedaan naar het exacte mechanisme van ontstaan. Omdat dit nog steeds niet duidelijk is, kan het ziektemechanisme worden gezien als een 'secret recipe'. In dit proefschrift wordt daarom gezocht naar nieuwe ingrediënten van het geheime recept van HUS.

Infectie-gerelateerde HUS

HUS wordt vaak veroorzaakt door een bacterie, genaamd *Escherichia coli* O157:H7. Deze bacterie komt voor in het darmstelsel van vee, zoals koeien en schapen. Deze dieren hebben er geen last van, maar kunnen de bacterie uitscheiden in hun ontlasting. Op deze wijze komt het bij de mens terecht: via besmetting van vlees in het slachthuis, via biologische voedingsmiddelen of via slechte handenhygiëne na bezoek aan dieren. Het meest voorkomend is besmetting via niet goed-doorbakken gehakt. Daarom wordt het ook wel de 'hamburger-ziekte' genoemd. De bacterie komt dus via voedsel in de darm van de mens en bindt hier aan de darm wand. Hiervoor gebruikt het een ingenieus mechanisme, waarbij het een eigengemaakt anker in de cel plaatst. Dit geeft een ontsteking van de darm waardoor diarree ontstaat. Deze kan bloederig zijn. Dat is een alarmsignaal waarbij altijd contact moet worden gezocht met een arts!

Nadat de bacterie is verankerd aan de darm kan het een toxine (=gifstof) gaan produceren. Deze wordt door de bacterie uitgescheiden en komt in het bloed terecht. Vanaf hier wordt het specifiek vervoerd naar de nier. Cellen in de bloedvaten van de nier zijn extra gevoelig voor dit toxine en gaan dood. Hierdoor ontstaat stolling in de nier en een massale ontstekingsreactie. Dit leidt ertoe dat de nier niet meer functioneert en hierdoor afvalstoffen niet meer uitgescheiden kunnen worden. Dit heet acuut nierfalen en behoeft snel handelen door middel van nierdialyse.

Zo omschreven lijkt het een duidelijk mechanisme, maar er zijn nog steeds veel vragen niet opgelost. In dit proefschift wordt ondermeer gekeken hoe het toxine zich kan verplaatsen in het bloed. Nog nooit is het toxine gevonden in het bloed van mensen met HUS. Hieruit kan geconcludeerd worden dat het ergens aan moet binden. Twee verschillende bloedceltypes zijn bestudeerd op een mogelijke functie als transporter. Ten eerste werd de neutrofiel bekeken. Dit is een witte bloedcel en betrokken bij de afweer. Deze cel bleek echter niet in staat om het toxine te binden en was ook niet gevoelig voor het effect van het toxine. Alle experimenten werden buiten het lichaam op gekweekte cellen (=*in vitro*) uitgevoerd. Hierbij moet dan altijd worden afgevraagd of dat betrouwbaar is om te vergelijken met de situatie in het lichaam (=*in vivo*). Omdat het niet verantwoord is om het toxine aan mensen te geven, werd het geïnjecteerd bij muizen. Maar ook bij deze experimenten konden we het toxine niet terugvinden aan de neutrofiel.

Een ander type witte bloedcel is de monocyt. Deze cel is wel in staat om het toxine te binden. Het is verder interessant om te vermelden dat deze cel niet doodgaat door het toxine, maar zelfs wordt geactiveerd. Maar ook deze cel bleek na meerdere experimenten niet geschikt om het toxine in het bloed te transporteren. De zoektocht naar deze vraag zal dus moeten worden gecontinueerd. Bovendien zitten er in het bloed naast bloedcellen ook allerlei eiwitten die een poging tot bestuderen waard zijn.

Een ander onderdeel van dit proefschrift beschrijft het exacte effect van het toxine op de gevoelige vaatwandcellen. Ook deze cellen zijn net als de monocyt, in staat om het toxine te binden en op te nemen. In dit proefschrift beschrijven we de exacte

route van het toxine in cellen van de humane nier. Deze route heeft tot gevolg dat het toxine een specifieke celstructuur, namelijk de ribosoom, kan beschadigen. Hierdoor gaat de cel dood. Maar waar eerst werd gedacht dat dit het enige effect van het toxine was, is door onze experimenten gebleken dat ook nog andere mechanismes plaatsvinden. Als het toxine binnendringt in de cel, is er een snelle respons door het uitscheiden van opslagblaasjes (genaamd Weibel-Palade bodies). Deze zijn gevuld met belangrijke stoffen die eerste hulp kunnen verlenen aan de cel door middel van het aantrekken van ontstekingscellen en het reguleren van stolling. Voor het aantrekken van de ontstekingscellen spelen de heparan sulfaat proteoglycanen (HSPG) een belangrijke rol. Dit zijn varen-achtige structuren die aan de buitenkant van de vaatwandcellen zitten. Wanneer HSPG worden verwijderd van de vaatwandcellen, zijn minder witte bloedcellen in staat om te binden. Dit betekent dat deze celstructuren een rol spelen in het ziektemechanisme.

Bovendien wordt de cel door het toxine geactiveerd tot het produceren van eiwitten die betrokken zijn bij de ontsteking en de stolling. Maar door de gelijktijdige beschadiging van de cel, is het erg moeilijk voor de cel om ze daadwerkelijk te maken. Bij een hoge dosis van het toxine gaan de cellen dood. Bij een lage dosis toxine, zullen na lange tijd deze eiwitten geproduceerd kunnen worden.

Bovenstaande bevindingen geven een nieuwe impuls aan de inzichten in HUS. Onze bevindingen van dit proefschrift staan samengevat in figuur 1. Deze gegevens zullen moeten worden vertaald in een betere behandeling.

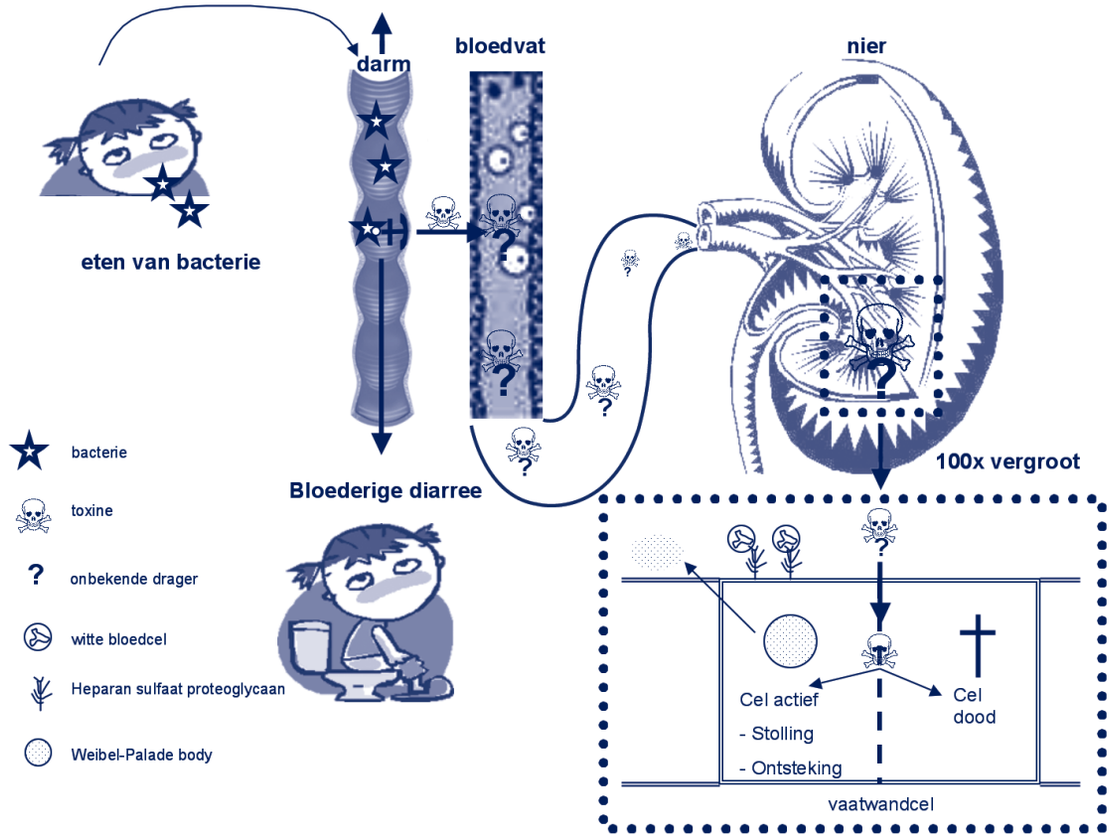
HUS door aangeboren defecten van het immuunsysteem

Het immuunsysteem van de mens is een zeer complex geheel. Een deel ervan is aangeboren en de rest moet worden gevormd gedurende het leven ten gevolge van het doormaken van verschillende infecties. Een onderdeel van het aangeboren systeem is het complementsysteem. Dit is een cascade van eiwitten die worden geactiveerd als een bacterie het lichaam binnendringt. Het complementsysteem is in staat om deze te doden door het maken van een gat in de wand van de bacterie. Het complementsysteem heeft een nauwe regulatie om te voorkomen dat het eigen lichaam wordt beschadigd. Deze regulatie bestaat uit eiwitten die gebonden zitten aan vaatwandcellen of circuleren in het bloed. Wanneer het systeem geactiveerd wordt door een bacterie en zich per ongeluk bindt aan een vaatwandcel, zijn deze regulerende eiwitten in staat om het fatale vervolg te stoppen. Echter, wanneer deze eiwitten niet goed functioneren, ontstaat er een beschadiging aan de vaatwandcellen specifiek in de nier. Dit leidt vervolgens tot het ontstaan van HUS.

Deze inzichten in het ontstaan van deze erfelijke vorm van HUS zijn zeer recent. In verschillende regulerende eiwitten van het complementsysteem zijn defecten gevonden. In dit proefschrift staat een nieuwe defect beschreven bij een patiënt met HUS, wat recidiveerde in zijn transplantatienier. Er bleek wel voldoende eiwit aanwezig te zijn, maar deze functioneerde niet naar behoren.

Door deze nieuwe inzichten is men nu in staat om te behandeling te optimaliseren. Er kan worden voorspeld op basis van het aangedane eiwit of er kans is van slagen van een niertransplantatie. Ook kunnen de defecte eiwitten in goede vorm worden toegediend aan de patiënt.

Figuur 1 Het ontstaansmechanisme van HUS



Curriculum Vitae, dankwoord & colofon

Curriculum Vitae
Joyce Geelen

Joyce Geelen was born on May 5, 1977 in the hospital of Almelo. She grew up in Weerselo, where she attended the Aloysiusschool. In 1995 she finished her secundary education at the Thijcollege in Oldenzaal. Unfortunately, she could not start the study Medicine because of numerus fixus. Instead, she started studying Health Sciences at the University of Maastricht. After finishing successfully her first year, she decided to start studying Medicine in Leuven, Belgium. In 1997, she could start her study Medicine at the Radboud University Nijmegen.
During her study Medicine she performed research at the department Paediatric Surgery (Prof. C. Festen) and Paediatric Nephrology (Prof L. Monnens). She received her degree (MD) in 2003 and started a PhD project at the Laboratory of Paediatrics and Neurology of the Radboud University Nijmegen Medical Center (promotor: Prof L. Monnens, co-promotor: dr. L. van den Heuvel). During this project, she visited for two months the laboratory of Prof H. Karch in 'Institut fur Hygiene' at the University of Münster, Germany. Furthermore, she performed shear-stress experiments at the laboratory of Prof. G. Remuzzi and Dr. M. Morigi at the Mario Negri Institute in Bergamo, Italy.
Since august 2007 she returned to work in the clinic at the Neonatal Intensive Care Unit of the Radboud University Nijmegen Medical Center (head: Dr. Liem). At this moment she is working at the Department of Paediatrics of Canisius Wilhelmina Hospital in Nijmegen (head: Dr. Gerrits).

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Colofon

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